Multi-Targeting of the mTOR Signaling Pathway is a Novel Therapeutic Strategy in Autosomal Dominant Polycystic Kidney Disease

DISSERTATION

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1 Zusammenfassung

Die polyzystischen Nierenerkrankungen (Englisch polycystic kidney disease; PKD) stellen eine Gruppe von monogenen Erkrankungen dar welche durch die Entwicklung von bilateralen Nierenzysten charakterisiert ist. Die häufigste Erkrankung ist die im Erwachsenenalter symptomatisch werdende autosomal-dominant polyzystische Nierenerkrankung (Englisch autosomal dominant polycystic kidney disease, ADPKD) während die juvenile autosomal-rezessiv vererbte PKD (ARPKD) seltener ist. Bei ADPKD geht die Entwicklung und Erweiterung der Nierenzysten mit Veränderungen des epithelialen Zellwachstums, der Flüssigkeitsabsonderung und der extrazellulären Matrixzusammensetzung einher. Diese Prozesse sind assoziiert mit Änderungen des mammalian target of Rapamycin (mTOR) - und des zyklisches Adenosinmonophosphat (cAMP)-vermittelten Signalwege. Diverse Substanzen wurden präklinisch und klinisch getestet, welche den mTOR oder den cAMP Signalweg beeinflussen mit dem Ziel den Krankheitsverlauf von ADPKD therapeutisch zu beeinflussen. Obwohl die Hemmung des mTOR-Signalwegs in präklinischen Studien erfolgreich den ADPKD Krankheitsverlauf verzögerte, waren die Ergebnisse der klinischen Studien mit mTOR-Inhibitoren, welche im New England Journal of Medicine veröffentlicht wurden, enttäuschend. Sirolimus und Everolimus, zwei mTOR Inhibitoren haben das Fortschreiten der ADPKD Erkrankung nicht gestoppt. Ich habe die möglichen Gründe für das Versagen der mTOR Inhibitoren in klinischen Studien mit zwei verschiedenen experimentellen Ansätzen eruiert: Erstens habe ich die Rolle des vorgelagerten Reglers von mTOR, die Phospholipase D (PLD) /

Phosphatidinsäure (PA) für PKD untersucht. Es wurde berichtet, dass PA, ein PLD Produkt, die mTOR-Aktivität reguliert. PA ist für die Stabilität des mTOR Komplex 1 (mTORC1) und des mTOR Komplex 2 (mTORC2) erforderlich und moduliert die Kinase Aktivität beider Komplexe. PA interagiert mit mTOR in einer Weise, die im Wettbewerb mit Sirolimus ist. Somit führt eine erhöhte PLD-Aktivität zu einer Resistenz gegenüber der hemmenden Wirkung von Sirolimus. Konstitutiv erhöhte PLD/PA-Signalwegaktivität wurde in einer Reihe von menschlichen Karzinomen, einschließlich Brust-, Eierstock-, Nieren-und Darmkrebs beobachtet. In Übereinstimmung mit diesen Ergebnissen, zeigte ich, dass die PLD-Aktivität in PKD Zellen abnormal erhöht ist, und teilweise zur Aktivierung des mTOR-Signalwegs beiträgt. Die pharmakologische Hemmung der PLD-Aktivität mittels PLD-Inhibitoren reduziert die Zellproliferation von PKD Zellen und blockiert den mTOR Signalweg, während exogenes PA den mTOR-Signalweg stimuliert und die hemmende Wirkung von PLD aufhebt. Ich konnte auch zeigen, dass die Blockierung der PLD-Aktivität die Empfindlichkeit der PKD Zellen für Rapamycin verbessert und dass die kombinierte Anwendung von PLD-Inhibitoren und Sirolimus synergistisch die PKD Zellproliferation hemmt. Zusätzlich konnte ich zeigen, dass mTOR Inhibitoren den Prozess der Autophagie nicht induzierte, während im Gegensatz dazu die Hemmung des PLD Signalweges die Bildung von Autophagosom stimulierte. Insgesamt deuten unsere Ergebnisse darauf hin, dass die Aktivierung des mTOR-Signalweges in PKD-Zellen teilweise durch erhöhte Aktivität des PLD-Signalweges vermittelt wird. Ob die Hemmung von PLD-Isoformen mit pharmakologischen Inhibitoren eine neue therapeutische Strategie für ADPKD darstellt, müssen weitere Untersuchungen zeigen.

In einem zweiten Ansatz habe ich meine folgende Hypothese geprüft: Die mTOR-Inhibitoren Everolimus oder Sirolimus wirken über eine duale Feedback-Schleife via des Phosphatidylinositol 3-Kinase (PI3K)-abhängigen Weges, der antiproliferativen Wirkung von mTOR-Inhibitoren in ADPKD entgegen. Ich konnte zeigen, dass die mTOR-Inhibitoren zur einer Blockierung der negativen Rückkopplungsschleifen führt und damit zu einer Verstärkung der pro-proliferativen Signalwege Phosphatidylinositol-3-Kinase (PI3K)-Akt und PI3K-extrazellulären Signal-regulierten Kinase (ERK) in PKD Ratten und Maus Modellen wie auch in mononukleären Blutzellen (PBMCs) von ADPKD-Patienten. Basierend auf diesen Ergebnissen habe ich das therapeutische Potenzial untersucht für ein multi-targting des mTOR-Signalweges und seinen assoziierten dualen Rückkopplungsschleifen. Die Blockade von mTOR und seine Rückkopplungsschleifen mittels des dualen PI3K/mTOR Inhibitors NVP-BEZ235 blockiert effizient die pro-proliferative Signale und führte zu einer Normalisierung der Nierenfunktion und Nierenmorphologie von PKD Tieren, einschliesslich im orthologen ADPKD Maus Modell. Molekulare Untersuchungen *in vitro* und *in vivo* zeigen, dass die mTOR/PI3K Doppelhemmung die Aktivierung des Akt und ERK-Signalweges verhindert, welche in der in der Gegenwart von einzelnen mTOR-Inhibition beobachtet wurde. Unsere Ergebnisse deuten darauf hin, dass die kombinierte Hemmung von PI3K/mTOR eine wirksame Behandlung für ADPKD Patienten sein könnte.

2 Summary

Polycystic kidney disease (PKD) represents a group of monogenic disorders that is characterized by renal cyst development. The most common polycystic disorder is the late onset form, autosomal dominant PKD (ADPKD), whereas the infantile form, autosomal recessive PKD (ARPKD) is much rarer. In ADPKD, cyst development and enlargement are associated with alterations in renal epithelial cell growth, fluid secretion and extracellular matrix composition[1]. These processes are connected to the impairments of mammalian target of rapamycin (mTOR)- and cyclic adenosine monophosphate (cAMP)-mediated signaling pathways [2 3]. Thus, several drugs have been tested to inhibit the mTOR or cAMP signaling pathways in ADPKD in order to halt disease progression [3 4]. Although targeting of the mTOR pathway effectively retards the disease progression in preclinical studies, two subsequent clinical trials which were published in the *New England Journal of Medicine* reported that the use of mTOR inhibitors sirolimus and everolimus did not to halt the progression of ADPKD [5 6].

In my PhD thesis I investigated the mechanisms of mTOR-driven cystogenesis in order to explain the disappointing results of the clinical trials, using two approaches. Firstly, I investigated the upstream regulator of mTOR, the phospholipase D (PLD) / phosphatidic acid (PA) pathway which modulates the mTOR pathway in PKD. It has been reported that PA, as one of the PLD products, binds with mTOR complex in a competitive manner with rapamycin [7]. PA is required for the mTOR complex formation and stabilization [8]. As a consequence, elevated PLD activity leads to rapamycin resistance. It has

been reported that a number of human carcinomas, including ovary, breast, colon and kidney cancers associated with the aberrant PLD/PA signaling [9, 10]. Consistent with cancer research results, I showed for the first time that PLD activity is abnormally elevated, and partly contributed to mTOR pathway activation in *in vitro* PKD models. Pharmacological inhibition of PLD activity by PLD inhibitors reduced PKD cell proliferation and blocked mTOR signaling, whereas exogenous PA stimulated mTOR signaling and abolished the inhibitory effect of PLD on PKD cell proliferation. We also showed that blocking PLD activity enhanced the sensitivity of PKD cells to rapamycin and that combining PLD inhibitors and rapamycin synergistically inhibited PKD cell proliferation. Furthermore, we demonstrated that blockage of mTOR activation did not induce autophagy, whereas targeting PLD induced autophagosome formation. Taken together, our findings suggest that dysregulated mTOR pathway activation is mediated partly by increased PLD signaling in PKD cells. Whether targeting of PLD isoforms with pharmacological inhibitors may represent a new therapeutic strategy in PKD needs to be studied further.

Secondly, I hypothesized that mTOR inhibition by the mTOR inhibitors everolimus or sirolimus triggered the dual feedback loop phosphatidylinositol 3-kinase (PI3K)-dependent pathway, which may counteract the mTOR inhibitory effect of mTOR inhibitors in ADPKD [11-13]. I demonstrated that mTOR inhibitors resulted in the removal of negative feedback loops and up-regulated pro-proliferative phosphatidylinositol 3-kinase (PI3K)-Akt and PI3K-extracellular signal-regulated kinase (ERK) signaling in rat and mouse autosomal dominant polycystic kidney models and peripheral blood

mononuclear cells (PBMCs) from ADPKD patients. Based on these results, we sought to evaluate the therapeutic potential for multi-targeting of mTOR and its dual feedback loop as a new approach to treat ADPKD. Blockade of mTOR and its feedback loop with the dual PI3K/mTOR inhibitor NVP-BEZ235 abrogated these pro-proliferative signals and showed effectively improved renal function and normalized kidney morphology compared with mTOR inhibitors alone in ADPKD rat model Han:SPRD and mouse orthologous model. Molecular research *in vitro* and *in vivo* indicated that dual mTOR/PI3K inhibition abrogated the up-regulation of Akt and ERK signaling pathways, otherwise observed in the presence of single agent mTOR inhibition. Our findings suggest that multi-targeted PI3K/mTOR inhibition may represent an effective treatment for ADPKD.

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4 Polycystic Kidney Disease

Polycystic kidney diseases (PKD) represent a group of monogenic disorders that are characterized by renal cyst development. The most frequent familial cystic diseases are: the common, late onset, autosomal dominant PKD (ADPKD) and the mainly infantile, autosomal recessive PKD (ARPKD) [1].

4.1 Autosomal Dominant Polycystic Kidney Disease (ADPKD)

ADPKD is an important cause of renal failure and results from a mutation in the *PKD1* or *PKD2* gene [14-16]. The disease is characterized by progressive enlargement of renal cyst and deterioration of kidney function, leading to renal failure beyond mid-life.

4.2 Clinical Description of ADPKD

ADPKD is one of the most common genetic renal diseases and, with an incidence of 1:400 to 1:1000, is typically diagnosed in adults,. It manifests in the 3rd to 4th decade of life, one half of the patients reaches end-stage renal disease (ESRD) at an average age of 58. Cyst development also occurred in the pancreas, seminal vesicles and liver in ADPKD patients. Renal cysts can even be diagnosed in utero or at birth and 2-5% of ADPKD patients present severe neonatal morbidity and mortality. Typical presenting symptoms in adults include flank pain, hypertension, hematuria, urinary tract infections, and renal colic [17].

Cyst growth is a key process that leads to the formation of cystic kidney in ADPKD. Fluid-filled cysts of sizes that can vary from a few mm to more than 10 cm are distributed throughout the renal cortical and medullary region [18].

The cystic-lining cells are highly proliferation and poorly differentiated tubular epithelial cells [19]. Inflammatory infiltration and interstitial fibrosis caused by increased fibroblasts and collagen are also observed in ADPKD kidneys.

4.3 Genetics

ADPKD is genetically heterogeneous with two genes identified, *PKD1* and *PKD2,* and the chromosomal locus are 16p13.3 and 4q21 respectively. *PKD1* accounts for ~85% of cases in clinically identified populations, whereas mutations in *PKD2* account for ~15% of them [20]. The renal and extrarenal manifestations are similar in both genetic forms, but mutations of *PKD1* are associated with a more severe clinical course, the average onset of ESRD in patients with *PKD1* mutations being 54.3 years which is 20 years younger than in patients with *PKD2* mutations [21].

PKD1 includes 46 exons distributed throughout a ~52 kb genomic segment. The *PKD1* genes encodes polycystin-1 (PC1), a large integral membrane glycoprotein with 4303 amino acids and an estimated molecular mass of ~460 kDa [16, 17]. PC1 includes 11 transmembrane domains and an extracelluar region consisting of a variety of domains, which in other proteins are associated with protein-protein and protein-carbohydrate interactions, and a short 197-aa intracellular C-terminus. This intracytoplasmic tail contains a G-protein-binding domain, playing an essential role in signal transduction [15, 22]. The mainly function of PC1 is a receptor and meanwhile it also involved in cell-cell and cell-extracellular matrix interactions (Figure 1).

PKD2 includes 15 exons, spans a ~68 kb genomic region and encodes polycystin-2 (PC2), which is also an integral membrane glycoprotein. PC2

contains 6 transmembrane domains and intracytoplasmic N- and C-termini. PC1 and PC2 physically interact by their C-termini [14]. The role of PC1 is a receptor or adhesion molecule, and PC2 is a non-selective cation channel that modulates intracellular calcium homoeostasis. PC2 activity is regulated by PC1, a process that constitutes the polycystin-signaling complex (Figure 1) [23, 24].

4.4 Autosomal recessive polycystic kidney disease

Autosomal recessive polycystic kidney disease (ARPKD) is caused by mutations of the *PKHD1* gene and much less common than ADPKD [25]. The main features of ARPKD are early-onset progression, progressive deterioration of renal function from childhood into early adult life [26].

4.4.1 Clinical Description

ARPKD has an incidence of 1:20,000 and is typically diagnosed during the neonatal period with greatly enlarged, echogenic kidneys. Most of those who survive the neonatal period (about 80%) are alive after 10 years, but 1/3 of them will developed ESRD [26]. Longer-term survivors often develop severe liver complications, portal hypertension, and hypersplenism from periportal fibrosis.

ARPKD renal cysts mainly originate from collecting ducts, presenting as fusiform, ectatic and dilated tubules which radiate from the medulla to the cortex. 10-90% of collecting ducts are affected and this is associated with increasing severity of renal disease. Unlike ADPKD, cystic lesions in ARPKD retain the structure of afferent and efferent connection [27].

4.4.2 Genetics

ARPKD is caused by mutation in *PKHD1* gene located on locus 6q21. *PKHD1* presents with a high level of allelic heterogeneity, around 40% of mutations are truncation and 60% missense mutations [28]. There is a genotype-phenotype correlation: two truncating mutations always associate with the most severe phenotype, neonatal death, whereas many missense changes are not fully penetrant alleles [25].

PKHD1 encodes fibrocystin, which contains a short intracellular C-terminal tail, a single transmembrance domain and a large extracellular N-terminal portion [29]. Fibrocystin has been found in the same complex as PC2, localizing to the primary cilia and the basal body [30]. The function of fibrocystin remains to be elucidated, but it might be a receptor protein and mediate its activity through PC2 (Figure 1) [31].

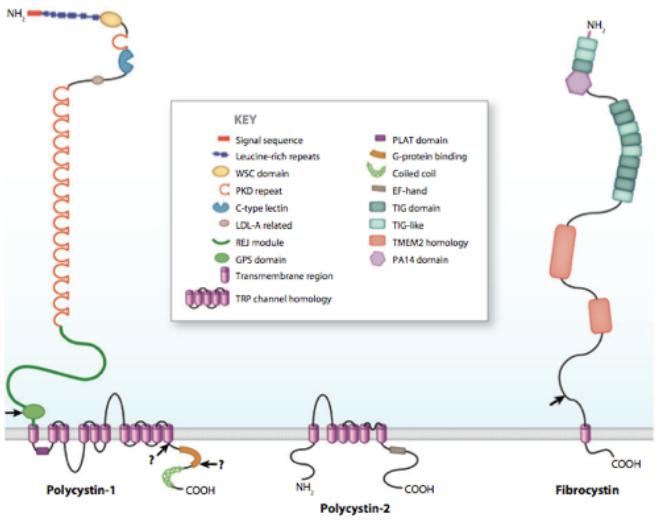

Figure 1. Structures of polycystin1, polycystin 2 and fibrocystin. Details of domains found in these proteins are shown in the key. Arrows indicate places where the proteins are thought to be cleaved. From Peter C. Harris (2009) [32].

5 Cellular Pathways Associated with PKD

Polycystins and fibrocystin modulate diverse cell signaling pathways and making the process of elucidating these molecular mechanisms is complicated. Such signalling pathways will be discussed in the following sections.

5.1 Ca^{2+}/cAMP Signaling

In general, the cyclic adenosine monophosphate (cAMP)-dependent pathway is a G protein-coupled receptor triggered signaling cascade, which, in turn, catalyzes the conversion of ATP into cAMP. The abnormal cAMP-dependent can lead to cellular hyper-proliferation.

In the kidney, the polycystin complex is thought to be a functional ion channel and also as a mechano/chemo-sensor in the cilia, translating extracellular stimuli into Ca^{2+} influx. Polycystin 2 controls the intracellular calcium homoeostasis through its function as an ion channel (Figure 2) [24]. Dysfunctional polycystin complex display altered intracellular calcium homoeostasis in PKD [33]. Defective Ca^{2+} signaling shown in cyst-lined cells that correspond to mechano-dependent cilia and reduced ER Ca^{2+} stores and ultimately decreased intracellular Ca^{2+} concentration [34]. The hypo-concentration of Ca^{2+} in the renal collecting ducts may lead to increase the intracellular cAMP level and promote the cAMP-dependent genes expression in PKD [35, 36]. The abnormal renal concentration of cAMP may lead to proliferation and fluid secretion of the cystic epithelial cells [37].

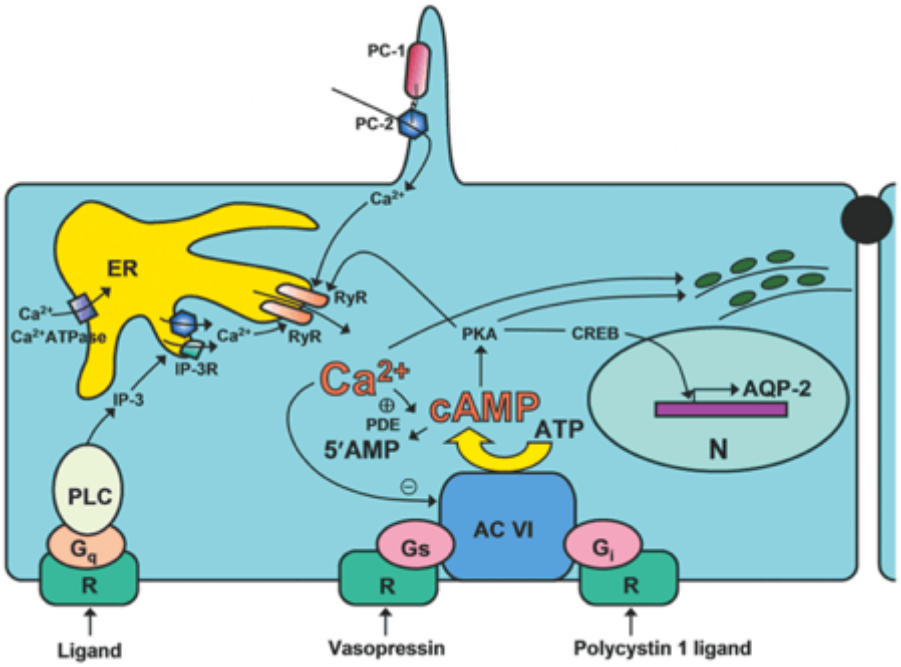

Figure 2. Polycystin complex modulates intracellular regulation of Ca^{2+} and cAMP in normal renal epithelial cells. Aberrant polycystin pathway reduced $[Ca^{2+}]_i$, and further stimulates adenylyl cyclase VI (AC VI) which leads to the blockage of cAMP-dependent phosphodiesterases (PDE) and as a consequence increased levels of intracellular cAMP. ER, endoplasmic reticulum; RyR, ryanodine receptor; R, G-protein-coupled receptor; PLC, phospholipase C; AQP-2, aquaporin-2; N, nucleus; PKA, protein kinase A; CREB, cAMP-responsive element binding protein. From Vincent HG (2003) [4].

5.2 Mammalian Target of Rapamycin

The discovery of rapamycin led to the identification of a highly conserved serine/threonine kinase, which named as mammalian target of rapamycin (mTOR). mTOR plays a master regulatory role in cell growth, proliferation and regulation of the cellular cytoskeleton and cell survival [38, 39]. Rapamycin forms a complex with the FK506-binding protein 12 (FKBP-12) and bind and inhibit mTOR assembly [40]. mTOR consists of two distinct complexes:

complex-1 (mTORC1) and complex-2 (mTORC2). mTORC1 defined by the binding of raptor, that regulates protein synthesis and cell proliferation. mTORC1 can be stimulated by growth factors, insulin, amino acids, phosphatidic acid and oxidative stress [41, 42]. mTORC2, assembled by the binding of rictor, is activated by growth factors [43]. mTORC2 regulates actin cytoskeleton and cell polarity [44].

5.2.1 Regulators and Effectors of mTORC1 and mTORC2

The lipid kinase phosphatidylinositol 3-kinase (PI3K) is a main regulator of mTORC1 signaling pathway, it activates serine/threonine kinase Akt by phosphorylation of Thr308 site. Akt further activates mTORC1 through a cascade of downstream intermediates, which include the tuberous sclerosis complex (TSC) and Rheb that directly activates mTORC1. The downstream targets of mTORC1 are the 4E-binding protein1 (4E-BP1) and the 70-kD ribosomal S6 kinases (p70 S6K). Activation of mTORC1 further phosphorylates the translation repressor protein 4E-BP1 at Thr37/46 site. Hyper-phosphorylation of 4E-BP1 binds to the translation initiation factor eIF4E and start the mRNA translation [45]. p70 S6K, as another downstream effector of mTORC1, is required for cell cycle progression and cell growth [46]. So far, little is known about the mTORC2 upstream regulation. Recently a study has shown that mTORC2 phosphorylates Akt at the serine residue S473 [47]. Serum- and glucocorticoid-induced kinase 1 (SGK1), which belongs to the same family of kinase as Akt, is also phosphorylated by mTORC2 [48].

5.2.2 Phospholipase D/Phosphatidic Acid and mTOR

Phosphatidic acid (PA), a short half-life small molecule, is one of the phospholipase D (PLD) products [7 8]. PA binds with mTOR complex in a competitive manner with rapamycin and is required for the mTOR complex formation. Hyper-activation of PLD may lead to rapamycin resistance. The aberrant PLD/PA signalling activity in human carcinomas is thought to promote cell proliferation via the mTOR and MAPK pathways (Figure 3) [9, 10].

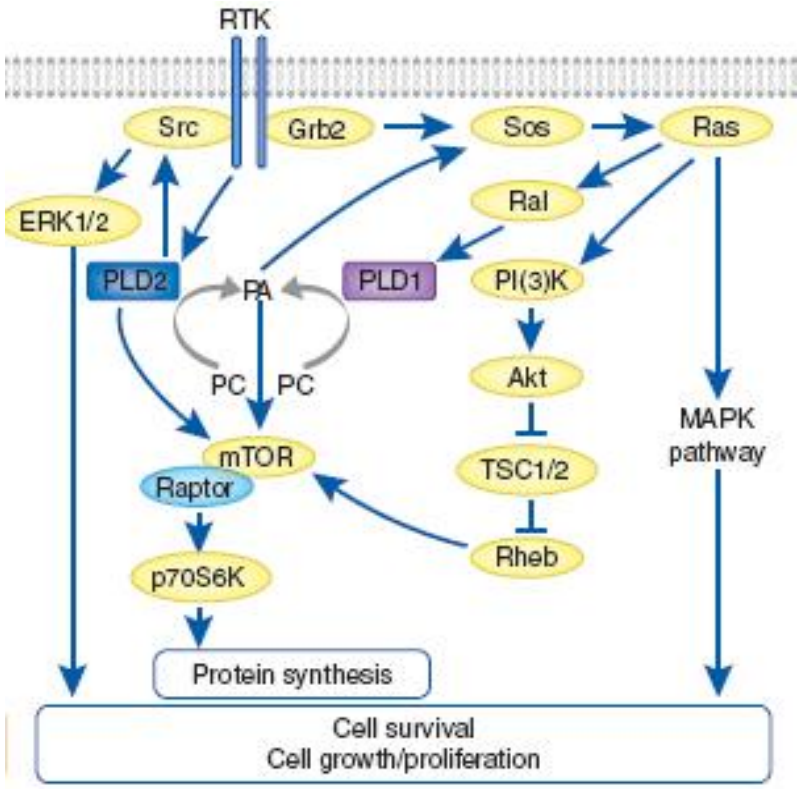

Figure 3. PLD/PA signalling pathway from receptor tyrosine kinase (RTK) to cellular response. From Sarah AS (2009) [49].

5.2.3 mTOR and Its Feedback Loop

A dual negative feedback loop has been reported in human cancer: mTOR/S6K activation attenuates upstream phosphatidylinositol 3-kinase

(PI3K) pathway activation, while treatment with mTOR inhibitors (rapamycin and its analogs) lead to a hyperactive insulin receptor substrate 1 (IRS-1)/PI3K pathway, and in turn increases the signaling toward to the pro-proliferative extracellular signal-regulated kinases (ERK) and Akt (Thr308 and Ser473 sites) pathways (Figure 4) [11-13].

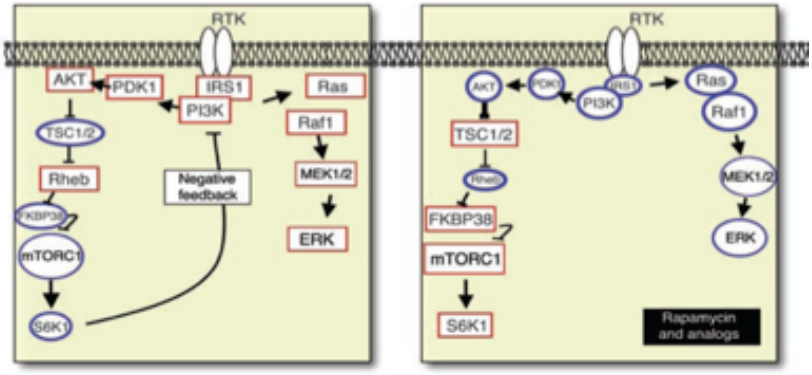

Figure 4. The mTORC1 pathway and its feedback loop. From Carracedo A (2008) [12].

5.2.4 mTOR Pathway in PKD

The cytoplasmic tail of PC1 interacts with tuberous sclerosis 2 (tuberin), a protein encoded by the tumor suppressor gene *TSC2*. It has been reported that tuberin acts as a Rheb GTPase-activating protein and inhibits mTOR activation. In rodent and human PKD, the dysfunctional polycystin complex leads to the hyper-activation of mTORC1 within the renal cystic epithelial cells. Also, PC1 induces the activation of mTORC2, which is responsible for phosphorylation of Akt Ser473, in cystic kidneys [3, 50, 51]. So far, there is no evidence of mTOR abnormal activation as a result of mutations in the *PKD2* gene.

5.3 Wnt Signaling

The Wnt signaling pathway is evolutionarily conserved, and functions across species ranging from the fruit fly to humans. Secreted glycoproteins of the Wnt family play an important role in the control of embryonic development and tissue regeneration. The two pathways, the canonical (β-catenin) pathway and the planar cell polarity (PCP) pathway received attention for their roles in multiple cellular processes within cystic kidney disease (Figure 5).

In the canonical Wnt pathway, the binding of Wnts to their receptors, Frizzled (Fzd) proteins, and the consecutive recruitment of the protein Dishevelled (Dvl) lead to an inactivation of the destruction complex, which promotes β-catenin accumulation and translocation to the nucleus and ultimately regulates the expression of target genes, including cell proliferation factors and proto-oncogenes as well as components of the Wnt pathway itself (Figure 5a). Wnt PCP signals can regulate epithelial-cell polarity across a sheet or tube, a process termed planar cell polarity. It also involves Fvd and Dvl receptors, but the downstream cascade differs in that it is independent of β-catenin stabilization, instead it activates the GTPases Rho and Rac as well as Jun N-terminal kinase (JNK) (Figure 5b).

PC1 seems to influence both the canonical and the PCP pathways. It has been found that hyper-activation of Wnt signaling pathway in PC1-knock down cells and cystic-lining cells which suggested that PC1 plays a negative role on this system [52, 53 54]. On the other hand, PC1 also regulates the Wnt PCP pathway by modulating cystic-lining cells deviation angle. The expansion of the tubule diameter in cystic-lining cells suggested that Wnt PCP pathway

maybe a key role for cyst formation in PKD [55, 56]. However, it is currently unclear whether PC2 directly modulates the Wnt pathway in PKD.

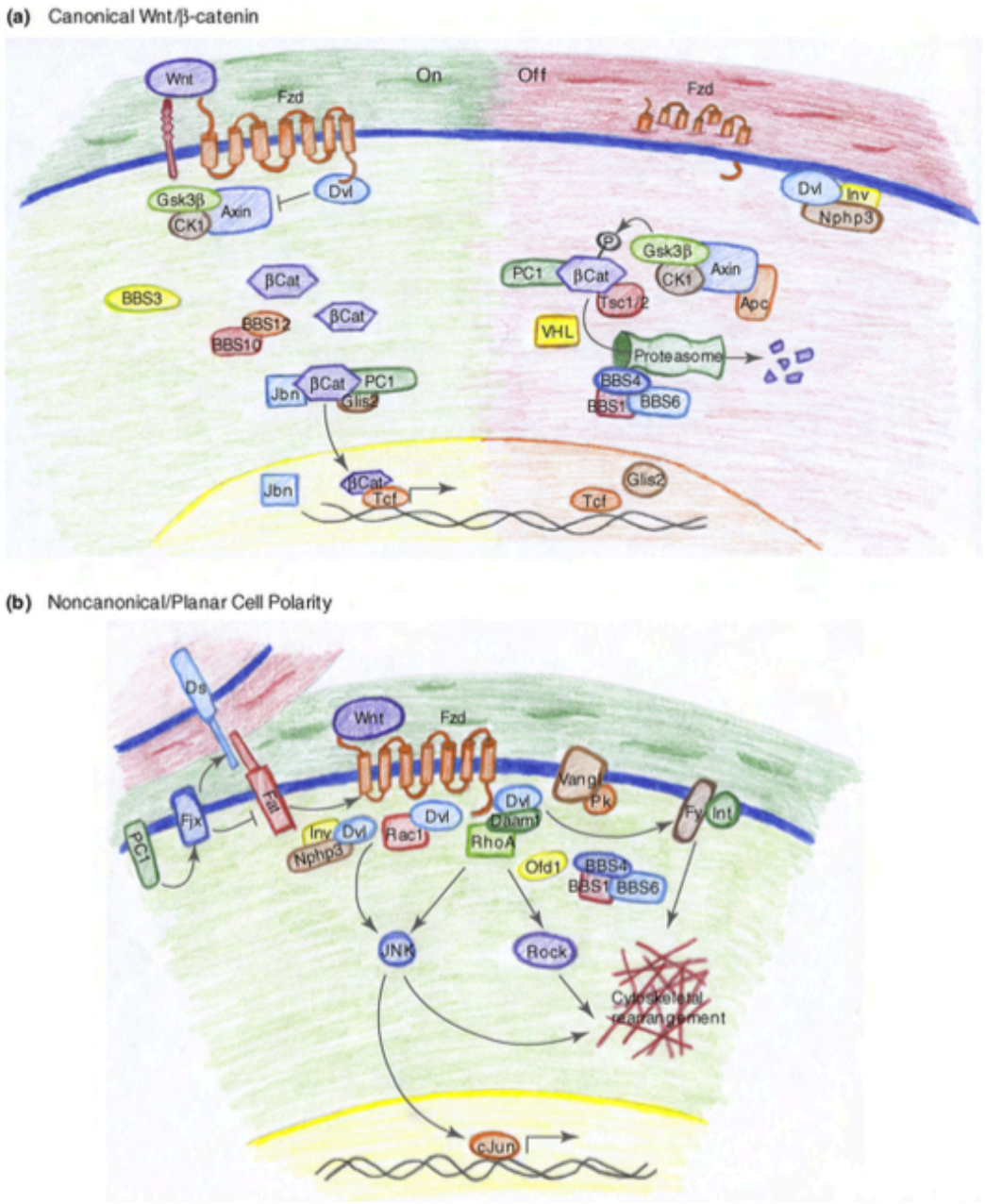

Figure 5. The Wnt pathways. (a) Drawing of the canonical Wnt/β-catenin pathway. In the ‘‘off’’ state (right), β-catenin (βCat) is targeted for degradation by phosphorylation by the destruction complex. When Wnt is present, the pathway is ‘‘on’’ (left), and the destruction complex is inhibited by Dvl, leading to the cytosolic accumulation of βCat. (b) Schematic of the PCP pathway. The PCP pathways might be activated by a Wnt ligand, followed by the activation of Frizzled (Fzd) and membrane-associated Dvl.

Dvl activates Ras homolog gene family member A (RhoA) or Ras-related C3 botulinum toxin substrate 1 (Rac1) to stimulate c-Jun N-terminal kinase (JNK) and Rho-associated kinase (Rock), which leads to downstream cytoskeletal rearrangements and transcriptional activation. From Madeline A (2010).

5.4 Mitogen-activated Protein Kinase/ Exracellular Regulated Kinase Signaling

The mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) is a highly conserved family of serine/threonine protein kinase and can be activated by a diverse range of extracellular stimuli through the activation of the Ras-Raf-MEK-ERK pathway, and crosstalk with many signaling pathways which involved PI3K/Akt, cAMP pathways. Activated MAPK/ERK modulates protein translation and regulates activities of several transcription factors to play an important role on cell cycle. Abnormal activation of ERK is observed in murine cystic models and cyst-derived cells from ADPKD patients [57, 58].

5.5 Janus Kinase/ Signal Transducer and Activator of Transcription Signaling

The Janus kinase/signal transducer and activator of transcription (Jak-Stat) signaling pathway utilized by a wide range of cytokines, growth factors and hormones. It controls the developmental regulation, growth control and homeostasis in organs [59]. The expression of these cytokines and growth factors is associated with PKD progression [60]. Furthermore, phosphorylation of STAT3 at Ser727 site is enhanced in mouse and human PKD [61]. It is likely

that the Jak-Stat pathway activation is, at least partially, required for the initiation and maintenance of cyst formation and growth in PKD.

6 Animal Models of PKD

The diversity of PKD experimental systems, especially the murine models (rat and mouse), provides us a good platform to elucidate the mechanisms of PKD progression. The clinical description of human PKD with respect to cyst development and renal function deterioration have been described in numerous murine PKD models

6.1 Rat Model

6.1.1 ADPKD Model

The Han:SPRD rat is a well characterized non-orthologous PKD strain which phenotypically resembles human ADPKD. The missense mutation in *Pkdr1* gene arose spontaneously in the Sprague-Dawley strain and is inherited as an autosomal dominant trait. The *Pkdr1* gene product, SamCystin, is a ciliary protein and is mainly distributed in the proximal tubules in which the initial cysts originate. Heterozygous cystic (Cy/+) Han:SRPD rats develop cystic lesions, which mainly localize in the proximal tubules, appear between 4 to 8 weeks of age, and keep increasing until 12 weeks of age [62]. Like human ADPKD, there is sexual dimorphism in disease expression. Renal function impairment is more pronounced in male heterozygotes than in female Cy/+. The strain also exhibits a gene-dose effect, as cystic homozygotes dead within week 3 [63]. There are numerous therapeutic interventions that have been evaluated in this model [64-66].

6.1.2 ARPKD Model

The PCK rat is an identified orthologous ARPKD model that can develop polycystic kidney and liver disease (PLD), which caused by a splicing mutation in the *Pkhd1* gene. The gene product, FPC is located in the cilia and expressed in the kidney, liver and pancreas [67]. Renal cystic lesions are observed in the distal tubules, thick ascending loops of Henle and collecting ducts after one week of age. There is also a sexual dimorphism in renal cystic disease expression similar with Han:SPRD rats, with male PCK rats develop more severe disease that the females [68].

6.2 Mouse Models

6.2.1 Non-orthologous Models

cpk mice

The *cpk* mouse caused by the spontaneously *cpk* gene mutation in the C57BL/6J strain. The *cpk* gene locates on chromosome 12 and encodes cystin which is localizes to the primary cilium. The *cpk* gene mutation translates to a recessive trait and resembling human ARPKD. Postnatal progressive cyst growth occurs predominantly in the collecting ducts leads to death around 3 to 4 week of age [69].

bpk mice

The BALB/c polycystic kidneys (*bpk*) mouse is an ARPKD model on the BALB/c inbred background caused by mutation of the homologous gene of

drosophila bicaudal C gene [70]. Affected homozygotes have a rapidly disease progression and dead within 4 weeks after birth.

jck mice

The juvenile cystic kidney (*jck*) mouse is an ARPKD model caused by juvenile cystic kidneys (jck) gene mutation.. The mutation gene *jck* encodes the Nek8 protein which localizes to the primary cilia and affects normal expression of polycystin complex. The mutants have slowly disease progression with 4 months of life span [71].

pcy mice

The *pcy* mouse is a model of ARPKD that is caused by a missense mutation in NPHP3. The gene product nephrocystin-3 is expressed in the primary cilia of epithelial cells in kidneys, liver, heart and pancreas [72]. Initially, cysts are derived from distal tubules, and whole nephron segments become diffusely occupied by cysts by 30 weeks of age. Death due to renal failure occurs between 30 and 36 week of age.

6.2.2 Gene-targeting Models Generated by Mutation of Human Orthologous Genes

Gene-targeting models are produced by deletion of human orthologous PKD genes, *PKD1* and *PKD2* for ADPKD, and *PKHD1* for ARPKD.

Pkd1 gene targeted mice

Because *PKD1* is the responsible gene in 85% of the patients with ADPKD, efforts have been made to generate *Pkd1* knockout mice with null or deleted exons. Heterozygous *Pkd1* targeting in mice results in slow, mild or even absent disease phenotype [73, 74]. However, almost all types of homozygously targeted *Pkd1* mice are lethal in the infant period [75]. The conditional knockout of the *Pkd1* gene using the Cre/loxP system results in a milder cystic phenotype which more appropriately reflects human ADPKD. The time period of conditional inactivation of the *Pkd1* gene influences the progression of ADPKD. Two studies reported that inactivation of *Pkd1* by tamoxifen before postnatal day 12 lead to severe cystic kidney disease within 3 weeks, whereas inactivation at day 14 or even later resulted in slowly progressing cysts formation after 5 month of age [76, 77]. Thus there seems to be a critical check-point which determines the severity of ADPKD progression.

Pkd2 gene targeted mice

The $Pkd2^{WS25/-}$ mouse is an ADPKD model with cysts in both kidney and liver [78]. It was established by crossbreeding of $Pkd2^{+/-}$ and $Pkd2^{WS25/+}$, and has a high level of proliferation and apoptosis in renal cysts.

Pkhd1 gene targeted mice

The homozygous *Pkhd1* gene targeted mice develop renal cysts from the collecting ducts and dilatation of the intrahepatic bile ducts in adulthood, resembling the PCK rat [79].

7 Therapeutic Strategies in PKD

No effective treatment is currently available for ADPKD. Since the identification of mutational genes PKD1 and PKD2, several compounds have been tested for therapeutic efficacy in ADPKD murine models and few drugs were applied in clinical trials. There are three major treatment strategies in PKD: (1) inhibit cell proliferation; (2) reduce cAMP levels, and (3) reduce fluid secretion.

7.1 Inhibition of Cell Proliferation

PKD has been described as “neoplasm in disguise” and thus an anti-proliferative therapeutic approach has become a popular research strategy.

7.1.1 mTOR inhibitors

The suggested activation of the mTOR pathway in PKD has prompted the testing of mTOR inhibitors. Administration of mTOR inhibitors (sirolimus or everolimus) in PKD rodent models has shown a beneficial effect on renal function and cyst growth [3, 80-82]. However, two clinical trials failed to shown a positive effect in either early or advanced disease stages [5] [6]. In the study by Walz et al., although total kidney volume was reduced, renal function did not improve but rather declined after 2-years everolimus treatment. The explanation of this disappointed result is might be the irreversibly damaged in the late stage of disease. In the study by Serra et al., 1.5 years sirolimus treatment in early phase ADPKD patients did not retard polycystic kidney growth.

It has been reported that mTOR inhibitors have a wide range of adverse effects, including stomatitis, bone marrow toxicity, hyperlipidemia, edema, and ovarian toxicity [83, 84]. Future research on mTOR inhibitors for ADPKD treatment needs to be performed to better define the therapeutic dosage, duration, time of intervention and alternative signaling pathways that maybe is stimulated by suppressing aberrant mTOR signaling activation.

7.1.2 Cyclin-dependent Kinase Inhibitors

Dysfunctional polycystin complex may directly affect the cell cycle and proliferation by promoting the activation of cyclin-dependent signaling pathway and resulting in renal cystic disease. Blocking cell cycle by roscovitine, a potent inhibitor of cyclin-dependent kinase 2 (Cdk2), inhibited cell proliferation, cystogenesis and improved renal function in *jck* and *pck* mouse model of PKD [85]. Furthermore, roscovitine has been shown to increase the level of p21, which is downregulated in PKD [86].

7.2 Therapies Reducing cAMP Levels

Abnormal elevated cAMP level has been observed in rodent ADPKD models and ADPKD patients that lead to the secretion of fluid into cysts and proliferation of cystic-lining cells [2]. These findings have provided a strong rationale for testing therapies targeting cAMP and cAMP signaling.

7.2.1 Vasopressin V2 Receptor Antagonist

The increased renal accumulation of cAMP maybe partly due to the activation of the vasopressin V2 receptor (VPV2R) pathway. Activation of this pathway

might be directly responsible for the increased cAMP levels and expression of cAMP-dependent genes which lead to proliferation of the cystic epithelium [4]. Thus, targeting VPV2R by its antagonist OPC-31260 reduced renal cAMP levels and inhibited cyst development in ARPKD and ADPKD animal models (Figure 2). OPC-31260 applied in PCK rats and *pcy* mice retarded disease progression and induced regression [4]. Tolvaptan, a highly selective antagonist for the human VPV2R, was also shown effective in PKD cellular models [87]. Recently, a phase III clinical trial with tolvaptan has been completed. The data show that when ADPKD patients receive tolvaptan for 3 years, total kidney volume growth was lower than in the placebo group [88]. The hazard ratio for worsening kidney function and kidney pain was in favor for tolvaptan-treated patients compared to placebo-treated study subjects. The drug-induced adverse effects were related to an increased excretion of electrolyte-free water (thirst, polyuria, nocturia and polydipsia) and elevated liver function tests [88]. The future use of this VPV2R antagonist will depend on a balance between benefits and risks.

7.2.2 Somatostatin Analogs

Targeting somatostatin receptors (SSTRs) decreases cAMP levels. Octreotide, a metabolically stable somatostatin analog, has shown effects on retarding cyst growth by reducing cAMP accumulation and the progression of liver and kidney cysts in PCK rats as well as in *in vitro* [89]. In a randomized, double-blind, placebo-controlled small clinical trial with 42 ADPKD patients, octreotide treatment halted kidney growth, whereas kidney volume increased in placebo-treated patients after two years treatment [90, 91]. Recently, Anna Caroli and

colleagues present the results of the ALADIN trial (A Long-Acting somatostatin on DIsease progression in Nephropathy due to autosomal dominant polycystic kidney disease), which was a multicentre, randomised, single-blind, placebo-controlled trial with the primary endpoint of change in total kidney volume at 1 and 3 year follow-up. At one year follow-up, the octreotide effect on kidney volume was impressive, however the inhibitory effect was greatly attenuated at year two and three. Notably, at study end, kidney and cyst volume were similar among octreotide and placebo treated patients. These data suggesting that octreotide administration may reduce kidney cyst growth only transiently. Adequately powered trials with longer treatment duration are needed to confirm the preliminary data [92].

Recently, a report shown pasireotide, a potent somatostain analog with broader kidney receptor specificity, is more efficient in reducing hepatorenal cystogenesis in PCK rats and *Pkd2*$^{WS25/-}$ mice [93].

7.2.3 Triptolide

Triptolide is a biologically active compound isolated from the medicinal plant *Tripterygium wilfordii*. Triptolide reduces cAMP levels by inducing intracellular calcium release and by inhibiting cystic-lining cell proliferation in *in vitro* and *in vivo* [94, 95]. Currently, a clinical trial of triptolide for ADPKD patients is ongoing in China.

7.3 Reduction of Fluid Secretion

Transporters required for chloride-driven fluid secretion into the cysts have been targeted to inhibit renal cyst growth.

7.3.1 Cystic Fibrosis Transmembrane Conductance Regulator Inhibitors

Cystic fibrosis transmembrane conductance regulator (CFTR) protein is a chloride/thiocyanate transporter. The mutations of CFTR lead to fluid accumulation and fibrosis in cysts. CFTR inhibitors retarded the cyst growth in *in vitro* and *in vivo* PKD animal models [96, 97]

7.3.2 Intermediate-conductance Ca^{2+}-activated K^+ Channel Inhibitors

An intermediate-conductance Ca^{2+}-activated K^+ channel (KCa3.1) inhibitor, TRAM-34, has been reported to inhibit transepithelial chloride secretion and cyst enlargement in ADPKD cells [98]. Senicapoc, a potent and selective blocker of human KCa3.1 channel, has been tested in a phase II and III clinical trial in sickle cell disease. That is encouraging to test the effect of KCa3.1 inhibitors in ADPKD animal models [99].

8 Purpose

Several line of evidence suggested that the mTOR signaling pathway plays a key role in PKD [3, 50, 51]. Numerous studies suggested that targeting the mTOR signaling by mTOR inhibitors effectively improved renal function and retarded cyst growth in a variety of murine models for PKD [76-78]. However, clinical trials vigorously testing efficacy of mTOR inhibitor therapy did not prove a beneficial effect on kidney growth and renal function [5 6]. Thus, in my PhD thesis I investigated the mechanisms of mTOR-driven cystogenesis in order to explain the disappointing results of the clinical trials, using two approaches.

Firstly, I investigated the upstream regulator of mTOR, the phospholipase D (PLD) / phosphatidic acid (PA) pathway which modulates the mTOR pathway in PKD. It has been reported that PA, as one of the PLD products, binds with mTOR complex in a competitive manner with rapamycin [7]. PA is required for the mTOR complex formation and stabilization [8]. As a consequence, elevated PLD activity leads to rapamycin resistance. It has been reported that a number of human carcinomas, including ovary, breast, colon and kidney cancers associated with the aberrant PLD/PA signaling [9, 10]. Secondly, I hypothesized that mTOR inhibition by the mTOR inhibitors everolimus or sirolimus triggered the dual feedback loop phosphatidylinositol 3-kinase (PI3K)-dependent pathway, which may counteract the mTOR inhibitory effect of mTOR inhibitors in ADPKD [11-13].

9 Published First Author Publication

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PLOS ONE

The Role of Phospholipase D in Modulating the MTOR Signaling Pathway in Polycystic Kidney Disease

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Abstract

The mammalian target of rapamycin (mTOR) signaling pathway is aberrantly activated in polycystic kidney disease (PKD). Emerging evidence suggests that phospholipase D (PLD) and its product phosphatidic acid (PA) regulate mTOR activity. In this study, we assessed *in vitro* the regulatory function of PLD and PA on the mTOR signaling pathway in PKD. We found that the basal level of PLD activity was elevated in PKD cells. Targeting PLD by small molecule inhibitors reduced cell proliferation and blocked mTOR signaling, whereas exogenous PA stimulated mTOR signaling and abolished the inhibitory effect of PLD on PKD cell proliferation. We also show that blocking PLD activity enhanced the sensitivity of PKD cells to rapamycin and that combining PLD inhibitors and rapamycin synergistically inhibited PKD cell proliferation. Furthermore, we demonstrate that targeting mTOR did not induce autophagy, whereas targeting PLD induced autophagosome formation. Taken together, our findings suggest that deregulated mTOR pathway activation is mediated partly by increased PLD signaling in PKD cells. Targeting PLD isoforms with pharmacological inhibitors may represent a new therapeutic strategy in PKD.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a inherited kidney disease characterized by progressive development of fluid-filled cysts in both kidneys, which results in end-stage renal disease in approximately 50% of affected individuals by the sixth decade of life. ADPKD is caused by mutations in the *PKD1* (approximately 85%) and *PKD2* (approximately 15%) genes encoding polycystin-1 and 2 (PC1 and PC2). PC1 and PC2 function in cell-cell and cell-matrix interactions, signal transduction and mechanosensation [1,2]. A direct physical interaction exists between the cytoplasmic tail of PC1 and the tumor suppressor tuberin, the product of the TSC2 gene that regulates the kinase activity of mTOR. Mutations in PC1 disrupt this interaction, unleashing mTOR and in turn, promote the proliferation of cyst-lining epithelial cells in ADPKD by aberrant signaling through mTOR [3]. .

mTOR is a Ser/Thr kinase that governs a wide variety of biological and cellular processes, including cell growth, proliferation, survival and autophagy [4,5]. mTOR is composed of two functionally and structurally distinct complexes: mTORC1 and mTORC2 [6]. The binding of raptor to mTOR defines the nutrient-sensitive mTORC1 that regulates protein synthesis by phosphorylating its substrates the 4E-binding protein1 (4E-BP1) and the 70-kD ribosomal S6 kinases (S6K) [7]. Rapamycin in a complex with its intracellular receptor FKBP12 specifically binds to the FKBP12/rapamycin binding domain of mTOR and inhibits mTORC1 function. mTORC2, assembled by the binding of rictor, a rapamycin-insensitive companion of mTOR, is activated by growth factors alone. The commonly described substrate of mTORC2 is Akt at the Ser473 site [8].

Phosphatidic acid (PA), a phospholipase D (PLD) product generated by the hydrolysis of phosphatidylcholine, regulates mTOR activity [9]. PLD is activated by a variety of hormones,

growth factors and cytokines. Two PLD isoforms are expressed in most mammalian tissues: PLD1 and PLD2, which are endowed with different properties, regulatory mechanisms and functions [10]. PA is required for the stability of mTORC1 and mTORC2 and modulates the kinase activity of both complexes. PA interacts with mTOR in a manner that is competitive with rapamycin. As a consequence, elevated PLD activity confers rapamycin resistance [11]. Aberrant PLD/PA signaling has been observed in a number of human carcinomas, including breast, ovary, kidney and colon cancer [12–14]. The elevated PLD activity in human carcinomas is thought to promote cell proliferation and to suppress the default apoptotic programs, thereby promoting cancer growth. We hypothesized that PLD activity governs PKD associated cell proliferation via the mTOR signaling pathway in PKD; however this has not been examined yet.

Autophagy, also called “self-eating”, is an evolutionarily conserved cellular pathway whereby cytosolic components are targeted for removal into membrane-bound compartments, named autophagosomes [15]. Autophagy has been well established as a cytoprotective mechanism under stress conditions, such as starvation. A number of studies have provided evidence that inadequate levels of autophagy can also lead to non-apoptotic cell death [15,16]. As mTOR signaling modulates autophagy and abnormally increased mTOR signaling is a feature of PKD, a connection between autophagy and PKD has been proposed [17]. However, there is so far only one report showing abnormalities in autophagy and autophagy-related proteins in PKD animal models [18].

In the current study, we show for the first time that PLD activity is abnormally elevated, and partly contributes to mTOR pathway activation in PKD cells. The mTOR signaling pathway is modulated in a PLD-dependent way in PKD. Inhibition of PLD activity increased the inhibitory effect of rapamycin on mTOR. Furthermore, targeting PLD impaired cell proliferation and induced autophagy, which may represent an opportunity for the development of new treatment strategies for PKD [19].

Materials and Methods

Antibodies and reagents

All commercial antibodies and chemicals were purchased from the following suppliers: anti-phospho-(T308)-Akt (4056), anti-phospho-(S473)-Akt (4051), anti-phospho-(T389)-p70 S6K (9206), anti-phospho-(T421/S424)-p70 S6K (9204), anti-p70 S6K (2708), anti-LC3B (3868), anti-caspase 3 (9662), anti-phospho-(S561)-PLD1(3834), anti-PLD1(3832), anti-phospho-(S235/236)-S6 (2211), anti-S6, anti-phospho-(T37/46)-4EBP1 (9459), anti-4EBP1(9644), anti-phospho-(S2448)-mTOR (2971), anti-mTOR (2983), anti-Atg5 (8540) antibodies were from Cell Signaling Technology; anti-Akt (ab8805-200) was from Abcam; anti-GAPDH (MAB374) was from Merck Millipore; anti-phospho-(Y169)-PLD2 (A8400) was from Assay Biotech; anti-PLD2 (sc18532) was from Santa Cruz; Sheep anti-mouse IgG-HRP (NA931A) and donkey anti-rabbit IgG-HRP (NA934A) were from GE Healthcare; Alexa Fluor 488 donkey anti-rabbit IgG (A21206) was from Life technology; 1,2-dioleoyl-*sn*-glycero-3-phosphoric acid monosodium salt (DOPA, 74304), rapamycin (R0395), honokiol (H4914), 1-BtOH (B7906) and *tert*-BtOH (19460) were from Sigma-Aldrich AG. PLD1 and PLD2 inhibitors were kindly provided from Prof. H. Alex Brown and Prof. Craig W. Lindsley.

Primary and immortalized renal tubular epithelial cell cultures

The Han: SPRD rat colony was established in our animal facility from a litter which was obtained from the Rat Resource & Research Center (Columbia, MO, USA) and kept under local regulation and guidelines. The animal study was approved by the animal health regulatory agency of the Canton Zürich, Switzerland. Heterozygous cystic (Cy/+) and wild-type normal (+/+) male rats, aged 8-weeks-old were used in this study. Primary renal epithelial cells were isolated as follows: kidneys were minced and digested by 1 mg/ml collagenase with gentle agitation for 1 hour at 37°C. The suspension was allowed to sediment for 1 minute twice. Cells were collected by harvesting the supernatant and then washed 3 times with 10% FBS/HBSS. Isolated cells were re-suspended in K1 medium (1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 5% FBS,10 mM HEPES, 42 mM sodium bicarbonate, 50 ng/ml insulin, 50 nM hydrocortisone, 50 ng/ml transferrin, 5 pM trijodothyronine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were seeded in collagen type 1-precoated culture dishes.

Primary cultures of normal human kidney epithelial cells (NHK) and ADPKD cyst-lining renal epithelial cells (ADPKD) were generated by the PKD Research Biomaterials and Cellular Models Core at the University of Kansas Medical Center (Kansas City, KS, USA) [20]. Human immortalized cystic (OX161) and noncystic (UCL93) cells were kindly provided by Prof. A.C. Ong (University of Sheffield, Sheffield, UK) [21]. The culture conditions of human primary and immortalized renal epithelial cells were the same as for rat primary renal tubular epithelial cells.

Cell viability and proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based CellTiter 96 AQueous One assay (G3581, Promega) was used to quantify cell viability. Cell proliferation was assessed using BrdU Cell Proliferation ELISA Kit (11647229001, Roche Applied Science), which quantifies cell proliferation by measuring DNA synthesis. The assay was performed according to the manufacturer’s instructions and results were expressed as mean absorbance of the samples measured in an ELISA plate reader.

Western blot analysis

Total cell lysates were prepared in the ice-cold lysis buffer containing 40 mM Hepes, 120 mM NaCl, 1 mM EDTA and 1% Triton (pH 7.5), supplemented with proteinase inhibitors (Roche) and phosphatase inhibitors (10 mM potassium pyrophosphate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate). The amount of protein was determined by using the BCA Protein Assay (23225, Thermo Scientific). Cell lysates in SDS-sample buffer

were boiled for 10 minutes at 95°C and equal protein amounts were resolved by SDS-PAGE, immunoblotted using polyvinylidene difluoride membranes and probed with antibodies. Signals were visualized by using the Chemiglow West chemiluminescence substrate kit (60-12596-00, ProteinSimple).

Preparation of PA

DOPA (74304, Sigma-Aldrich AG) was dissolved in chloroform and dried under nitrogen. The lipid film was resuspended in vesicle buffer (150 mM NaCl and 10 mM Tris-Cl (pH8.0)) by vortexing briefly to yield a final lipid concentration of 10 mM. The lipid suspension was then sonicated in a water bath sonicator for 5 min. This procedure is expected to yield small unilamellar vesicles with diameters in the range of 15-50 nm. Lipid vesicles were made freshly before each experiment and were added directly to cell medium at the final concentration of 100 μM. Due to the short half-life of PA, this process was repeated every 40 min throughout treatment.

PLD enzyme activity

The protein samples were collected and the concentration determined using the same procedures as describes for the Western blot assay. 50 μg of protein samples were used to determine PLD activity with the Amplex Red PLD assay kit (A12219, Invitrogen), according to the manufacturer's protocol.

Immunofluorescence staining

Cells were seeded into 6-well plates with coverslips. 24 h later, cells were treated with or without PLD inhibitors for 48 h. Cells grown on the coverslips were fixed and permeabilized with ice-cold methanol at -20°C for 10 min. After 3 washes with PBS, the samples were blocked with blocking buffer (1% BSA, 1% Triton X-100 in PBS (pH 7.4)) at room temperature for 30 min. The samples were then incubated with primary antibody (anti-LC3B) at room temperature for 2 h. After 3 washes with PBS, the samples were incubated with Alexa Fluor 488 donkey anti-rabbit IgG at room temperature avoiding light for 1 h. Slides were examined by using a laser scanning confocal microscope (Leica SP5).

Transmission electron microscopy

ADPKD and OX161 cells treated with or without either 10 μM PLD1 inhibitor or 20 μM PLD2 inhibitor for 48 h were fixed for 2 h at room temperature (RT) with 2.5% glutaraldehyde in PBS (pH 7.4) and, subsequently, with 1% OsO4 in 50 mM sodium cacodylate buffer (pH 7.3), dehydrated in an ethanol series and embedded into epon (Catalyst). Ultrathin sections of 50 nm were contrasted with uranyl acetate and lead citrate and analyzed in a Tecnai Spirit transmission electron microscope (FEI) with an ORIUS CCD camera (Gatan).

Statistical analyses

Statistical analyses were performed by one-way ANOVA with the Dunnet post-hoc test. All data are expressed as means ± SD. P values were two sided for the comparison between the groups or between baseline and follow-up values, and those less than 0.05 were considered statistically significant.

Results

Elevated mTOR and PLD activity in PKD cells

In this study, we used 3 different PKD cellular models: 1) primary renal tubular epithelial cells derived from heterozygous Cy/+ and +/+ Han: SPRD rats, a well characterized strain (Cy/+), which phenotypically resembles human ADPKD [22]; 2) an immortalized human ADPKD renal tubular epithelial cell line (OX161) and an immortalized normal renal tubular epithelial cell line (UCL93) [21]; and 3) primary renal epithelial cells derived from ADPKD patients (ADPKD) and from normal human kidney tissue (NHK) [23]. Western blots analysis was used to examine the expression of regulators and effectors of mTOR complexes in the PKD renal cystic epithelial cells, and were compared with normal renal tubular epithelial cells. Figure **1A** shows the markers of activation of mTORC1 (phosphorylation at T308 of Akt, and at T389 of S6K) and mTORC2 signaling pathway (phosphorylation at S473 of Akt). Mutations in *PKD1* or *PKD2* lead to profound effects on downstream target tuberous sclerosis complex (TSC)-mTOR pathway in ADPKD. In our study, the basal activity of mTOR, assessed by the phosphorylation status of the mTOR readouts Akt and S6K, was higher in the primary PKD cells, compared with normal renal epithelial cells. In the immortalized cells, we observed that both OX161 and UCL93 cells had high activation of Akt and S6K, which suggests that the elevated mTOR pathway activity is associated with increased cell proliferation in immortalized PKD and normal cells.

Since it was recently shown that aberrant PLD activity is associated with several human cancers, and PA, a product of PLD activation, is required for the stability and activity of the mTOR complex, we hypothesized that the abnormal mTOR pathway activation could be partly due to high basal PLD activity in PKD. To test this hypothesis, we examined endogenous PLD activity by measuring its activity *in vitro*. In this study, we used honokiol to treat OX161 as a positive control when measuring PLD activity with the specific enzymatic assay [24]. Honokiol, a bioactive compound obtained from several species of the genus *Magnolia* of Magnoliaceae family, suppressed the PLD activity through targeting the activation of the upstream regulator Ras [25]. The data presented in Figure **1B** shows that the basal level of PLD activity is increased in all investigated PKD cell models compared with normal renal epithelial cells and that honokiol decreased PLD activity of OX161 cells.

PLD Inhibitors Impair Cell Proliferation in Renal Tubular Epithelial Cells

Since up-regulated PLD activity was observed in all PKD cells we hypothesized that targeting PLD could be an effective treatment strategy for PKD. We therefore examined the efficacy of specific inhibitors of PLD1 and PLD2 on PKD cell proliferation which was determined by MTS, BrdU and cell counting assays [26]. The PLD inhibitors we used as Scott SA et al. reported that directly interact with the catalytic domains of

Fig. 1

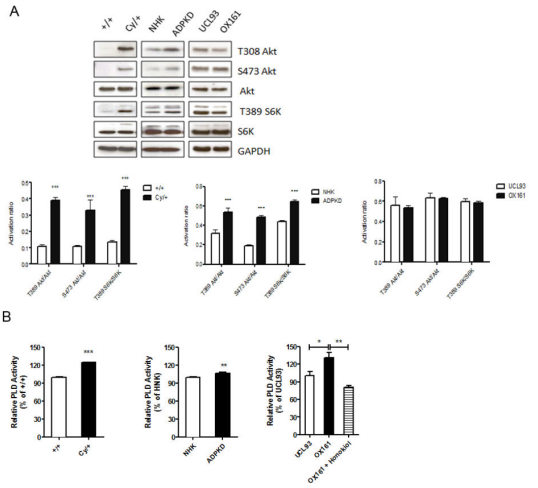

Figure 1. Elevated mTOR and PLD activity in PKD cells. (A) Western blots and densitometry analyzing the endogenous activity of the mTOR in PKD and control cells. (B) The endogenous level of PLD activity was determined in PKD and control cells by the Amplex Red PLD assay kit. Honokiol (20 μM) treated OX161 for 20 h as a positive control. An experiment which is representative of three independent experiments is shown. Data are expressed as mean ± SD and were analyzed by one-way ANOVA. * p< 0.05, ** p< 0.01, *** p< 0.001.

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the enzyme, and do not require binding with the regulatory PX-PH domain to execute inhibition [26]. Figure **2** shows that both PLD1 and 2 inhibitors reduced cell viability, DNA synthesis and cell proliferation in normal and PKD cells in a dose-dependent way. Both PLD inhibitors proved cytotoxic in all cell types and the PLD1 inhibitor exhibited a higher efficacy in reducing cell proliferation compared with the PLD2 inhibitor.

PLD inhibitors specific target PLD

We next focused our study on human PKD cells to examine whether PLD inhibitors could specifically block PLD phosphorylation. Western blot analysis indicated that the dose-dependent effect of PLD inhibitors with the reduced phosphorylation levels of PLD1 and PLD2. Figure **3A** shows that both PLD1 and PLD2 inhibitors reduced specifically the phosphorylation of PLD1 and PLD2, respectively. Using an enzymatic assay to correlate the inhibition of phosphorylation with PLD activity we found that both PLD inhibitors reduced total PLD activity in ADPKD and OX161 cell lysates (Figure **3B**). Inhibition of PLD activity was dose-dependent and maximal at doses of 10 μM and 20 μM for the PLD1 and PLD2 inhibitor, respectively.

Fig. 2

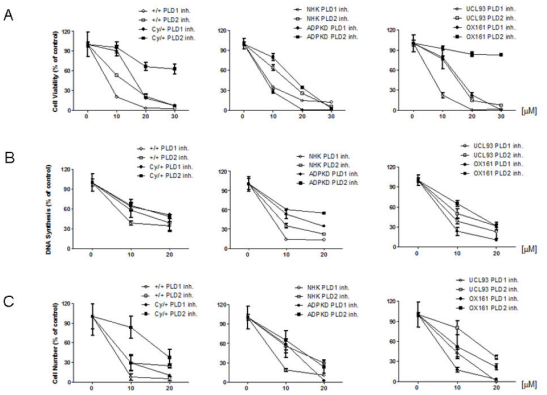

Figure 2. PLD inhibitors impair cell growth and block PLD activity in PKD cells. Effect of PLD1-/2-inhibitors on (A) the cell viability, (B) DNA synthesis ability and (C) cell proliferation were determined by MTS, BrdU and cell counting, respectively. All types of cells were exposed to various concentrations of either PLD1 or PLD2 inhibitor for 48 h. An experiment which is representative of three independent experiments is shown.

doi: 10.1371/journal.pone.0073173.g002

PLD inhibitors affect the mTOR signaling pathway, while exogenous PA stimulates mTOR signaling

PA is known to mediate the activation of mTOR signaling by binding to the FKBP12-rapamycin binding (FRB) domain of mTOR. Since we found that the activity of PLD was elevated in PKD, we tested the hypothesis that PLD activity is required to sustain the activation the mTOR signaling pathway in human PKD cells. Thus we treated ADPKD and OX161 cells with PLD1 and PLD2 inhibitors and examined their effect on the phosphorylation of the up- and down-stream targets of the mTOR pathway. We found that the phosphorylation of both the up-stream targets of mTORC1 (phospho-T308-Akt) and the down-stream targets (phospho-T421/S424-S6K, phospho-S235/236 S6, phospho-T37/46-4E-BP1) decreased in a dose-dependent manner (Figure **4A**). The readout for mTORC2, phospho-S473-Akt, also decreased upon PLD1 and PLD2 inhibitor treatment (Figure **4A**). To further assess the specificity of PLD inhibitors to block the mTOR pathway, we performed the “alcohol trap” assay. This alternative method of inhibiting PLD activity takes advantage of the fact, that PLD preferentially utilizes primary alcohols (1-BtOH) in the transphosphatidylation reaction, producing phosphatidyl alcohols instead of PA; whereas tertiary alcohols (*tert*-BtOH) is not a substrate for PLD and is therefore used as a negative control [27]. As shown in Figure 4B, 1% 1-BtOH suppressed the phosphorylation T421/S424-S6K and S473-Akt, whereas 1% tert-BtOH had no effect on the phosphorylation status, indicating that the observed, effects of PLD 1 and 2 inhibitors on the mTOR pathway were due to the suppression of PA production by PLD. As a second alternative method to block PLD activity, we used honokiol,

Fig. 3

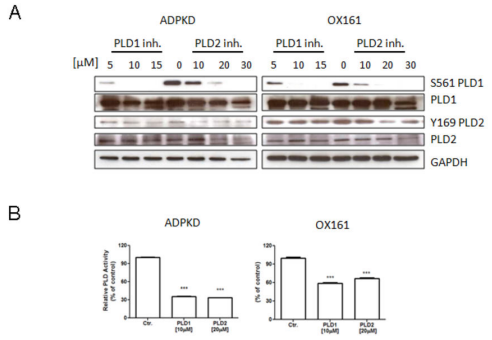

Figure 3. PLD inhibitors block the PLD/PA pathway. (A) Western blots analyzing the expression of phospho-PLD1, PLD1, phospho-PLD2, PLD2 and GAPDH either upon treatment with different concentrations of PLD1-/2- inhibitor (for PLD1 inhibitor: 5, 10, 15 μM, for PLD2 inhibitor: 10, 20, 30 μM) treatment for 48 h or without treatment. (B) PLD enzyme activity assay determining PLD activity upon treatment with either 10 μM PLD1 inhibitor or 20 μM PLD2 inhibitor for 48 h. An experiment which is representative of three independent experiments is shown. Data are expressed as mean ± SD and were analyzed by one-way ANOVA. ***p < 0.001.

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Fig. 4

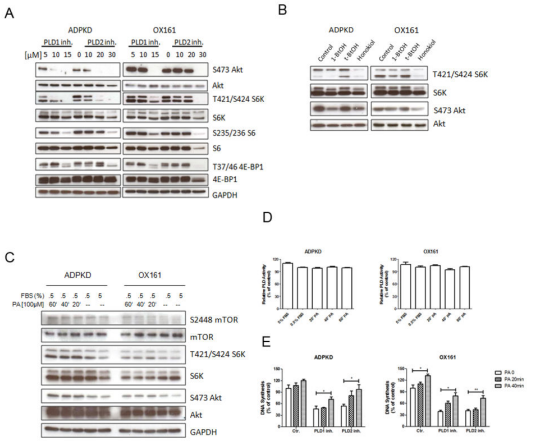

Figure 4. PLD inhibitors block mTOR signaling pathway and exogenous PA abrogates the effect of PLD inhibitors on mTOR signaling and proliferation of PKD cells. (A) PLD inhibitors affect mTOR signaling in a dose-dependent way. Western blots analyzing the expression of phospho-Akt, Akt, phospho-S6K, S6K, phospho-S6, S6, phospho-4EBP1, 4EBP1 and GAPDH either upon treatment with the indicated concentration of PLD1-/2- inhibitor for 48 h or without treatment. (B) PKD cells were plated for 24 h and then shifted to medium containing 0.5% serum. 1% 1-BtOH or 1% *tert*-BtOH was added for 2 h, 20 μM honokiol was added for 20 h. Western blots analyzing the expression of phospho-S6K, S6K, phospho-Akt and Akt. (C, D) Exogenous PA stimulated mTOR signaling in a time-dependent way, but not PLD activity. Human PKD cells were cultured under either normal culture medium (5% FBS) or starvation culture medium (0.5% FBS). After 24 h starvation, cells were treated with 100 μM PA for 20, 40 and 60 minutes. Whole cell lysates were analyzed by (C) western blots to detect the expression of phospho-mTOR, mTOR, phospho-S6K, S6K, phospho-Akt, Akt and GAPDH. (C) Whole cell lysates were determined by Amplex Red PLD kit to measure the PLD activity. (E) Exogenous PA impairs the effect of PLD inhibitors on cell growth. Cells were pretreated with either 10 μM PLD1 inhibitor or 20 μM PLD2 inhibitor for 48 h, and then with 100 μM PA for the indicated time. DNA synthesis was measured by using the BrdU assay. An experiment which is representative of three independent experiments is shown. Data are expressed as mean ± SD and were analyzed by one-way ANOVA. * p< 0.05, ** p< 0.01.

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considered as PLD1 and PLD2 inhibitor, to treat PKD cells. Figure 4B and Figure S1 shows, that also honokiol suppressed the phosphorylation of S6K and Akt in human ADPKD and OX161 cells. Taken together, our studies indicate that PLD 1 and PLD 2 inhibitors suppress mTOR signaling in PKD cells by decreasing specifically PLD activity.

To study the functional relevance of the inhibitory effect of PLD inhibitors on PLD activity, we examined the effect of bypassing the need for PLD activity on both mTOR signaling and cellular proliferation by adding exogenous PA to the cell culture medium. Because exogenous PA has a short half-life, we analyzed three short time points (20 min, 40 min and 60 min) of treatment with PA in both ADPKD and OX161 cells. We found that exogenous PA (100 μM) was able to stimulate by itself the activation of mTOR (S2448), S6K (T421/S424), Akt (S473 and T308) (Figure **4C**) without affecting PLD activity (Figure **4D**). Importantly, exogenous PA increased DNA synthesis in human PKD cells, and rescued the inhibitory effect of PLD inhibitors in a time-dependent manner (Figure **4E**). These results suggest that the mechanism of action of PLD inhibitors on cellular proliferation is via inhibition of PLD-dependent PA production and is partly mediated through an inhibition of the mTOR signaling pathway.

PLD inhibitor triggers autophagosome formation

mTOR is a master regulator of cell proliferation and modulates apoptosis and autophagy in many types of cells [4] [15]. To test whether PLD inhibitors affect downstream targets of mTOR, we examined the apoptosis and autophagy pathways after treatment with either PLD inhibitors, 1-BtOH, *tert*-BtOH or honokiol in ADPKD and OX161 cells. As shown in Figure **5A**, cleaved caspase-3, a marker of the apoptotic pathway, did not show an increased expression upon treatment. But immunoblotting analysis showed a drastic

conversion of non-autophagic soluble LC3 (LC3-I) to autophagic LC3 (LC3-II) in response to PLD1- and PLD2-specific inhibitor and honokiol treatment in a time- and dose-dependent manner (Figure **5A**). One pathognomonic feature of autophagy is the ultrastructural evidence of autophagosomes. We examined PLD inhibitor-treated ADPKD and OX161 cells using transmission electron microscopy to visualize the induction of autophagy. Figure **5B** shows that after 48 h exposure to 10 μM PLD1- and 20 μM PLD2-inhibitors autophagosomes were abundantly present. To further examine the formation of autophagic vesicles, we performed immunofluorescence analysis for LC3. As expected, LC3 aggregated substantially in response to PLD inhibitors in both ADPKD and OX161 cells (Figure **5C**).

Combinations of mTOR and PLD inhibitors have a synergistic effect on blocking the mTOR pathway in PKD cells

The therapeutic targeting of mTOR in PKD has attracted attention in recent years due to a link between mTOR and survival signals in human PKD [28–30]. However, clinical trials with rapamycin and rapamycin analogues have been disappointing [31,32]. We hypothesized that the lack of efficacy could be due to elevated PLD activity that may had conferred resistance to rapamycin [33]. To examine whether blocking PLD activity leads to a decrease in the cellular resistance to rapamycin, we treated ADPKD and OX161 cells with a combination of low dose rapamycin and PLD inhibitors. The combinatorial treatment of rapamycin and PLD inhibitors exhibited more efficacy in blocking the phosphorylation of the S6 protein compared with PLD inhibitors treatment alone in ADPKD cells, but not in OX161 cells (Figure **6A**). Furthermore, we investigated whether the combination treatment had a synergistic effect in inhibiting PKD cell proliferation. As shown in Figure **6B**, neither 5 μM PLD1- nor 10 μM PLD2-inhibitor alone had a marked effect on cell proliferation. However, the combination treatment of 0.1 nM rapamycin with PLD1- or PLD2-inhibitors inhibited cell proliferation in both ADPKD and OX161 cells.

mTOR has been shown to negatively regulate autophagy upon nutrient limitation, which leads to suppressed mTOR pathway activity in many cell types. Therefore, we examined whether combining mTOR and PLD inhibitors had a synergistic effect on inducing autophagy. A Western blot analysis showed that LC-II conversion did not increase in response to the combination treatment compared with single agent treatment (Figure **6A**). We further assessed whether the low dose of rapamycin (0.1 nM) was not sufficient to induce autophagy in PKD cells. To this aim, we treated ADPKD and OX161 cells with a range of rapamycin concentrations for 24 h. Interestingly; even the highest concentration of rapamycin did not trigger autophagosome formation in PKD cells (Figure **6C**). However, the phosphorylation of the ribosomal S6 protein was completely blocked by the rapamycin treatment under these conditions (Figure **6C**) indicating successful blockage of the mTOR signaling pathway. Our results suggest that PKD cells are resistant to rapamycin-induced autophagy. Taken together, these results show that PLD inhibitors can reduce the

Fig. 5

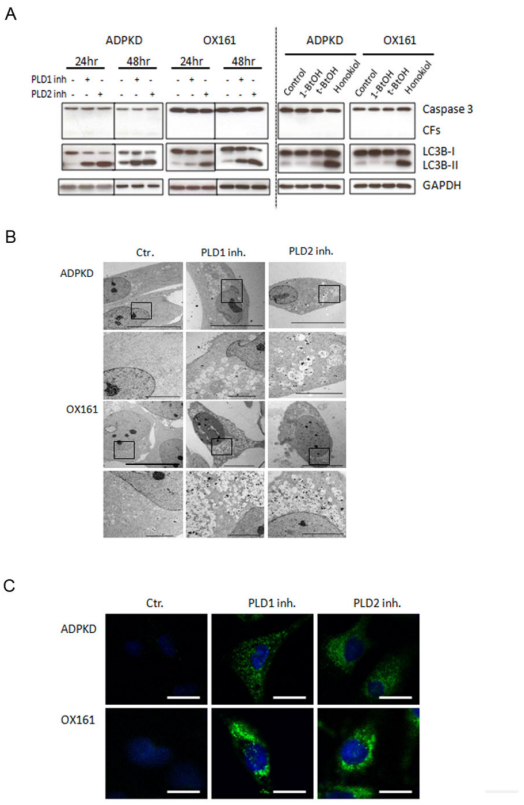

Figure 5. PLD inhibitors trigger autophagosome formation. (A) PLD inhibitors induced autophagy in a time-dependent way. Left panel: PKD cells were treated by either 10 μM PLD1 inhibitor or 20 μM PLD2 inhibitor for 24 h and 48 h. Right panel: PKD cells were plated for 24 h and then shifted to medium containing 0.5% serum. 1% 1-BtOH or 1% *tert*-BtOH was added for 2 h, 20 μM honokiol was added for 20 h. Western blots analyzing the expression of caspase-3, LC3B and GAPDH. (B) Transmission electron microscopy analysis of PLD1 and PLD2 inhibitor-treated HAK and OX161 cells (10 μM and 20 μM respectively, 48 h) showing intensive vacuolization and autophagosome-like vesicles. The lower panels show the magnification of the black frame area in the upper panels for each type of cell. The scale bar in the upper and lower panels represents 10 μm and 5 μm respectively. (C) Immunofluorescence microscopy analyzing autophagosome distribution after PLD1-/2- inhibitor (10 μM and 20 μM) treatment for 48 h. Cells were stained with anti-LC3B antibody. Scale bar represents 20 μm. Representative of three independent experiments.

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Fig. 6

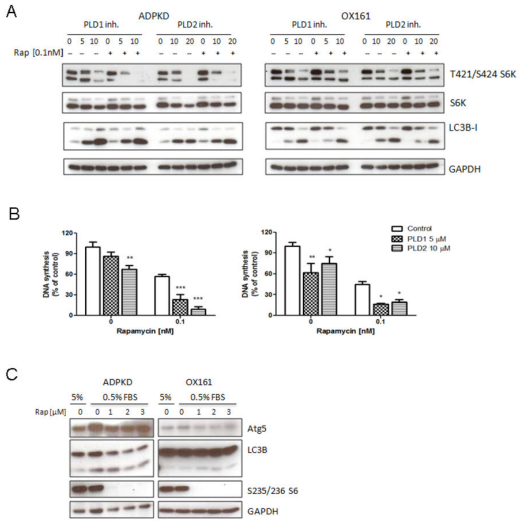

Figure 6. A combination of mTOR and PLD inhibitors has a synergistic effect on the mTOR pathway. (A) Combined mTOR and PLD1-/2-inhibitor treatment has a synergistic effect on blocking mTOR signaling in PKD cells. Western blots analyzing the expression of phospho-S6K, S6K, LC3B and GAPDH upon treatment with or without the indicated concentrations of PLD1-/2-inhibitor combined with 0.1 nM rapamycin for 48 h in PKD cells. (B) Combination treatment of mTOR and PLD inhibitors has a synergistic effect on blocking cell proliferation in PKD cell. BrdU assay determining DNA synthesis in human PKD cells upon treatment with or without the indicated concentration of PLD1-/2-inhibitor combined with 0.1 nM rapamycin for 48 h. (C) PKD cells display resistance to rapamycin-induced autophagy. Western blots analyzing the expression of Atg5, LC3B, phospho-S6 and GAPDH upon treatment with or without various concentration of rapamycin (1, 2, 3 μM) for 24 h under starvation conditions (culture medium containing 0.5% FBS). An experiment which is representative of three independent experiments is shown. Data are expressed as mean ± SD and were analyzed by one-way ANOVA. * p< 0.05, ** p< 0.01, *** p< 0.001.

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resistance of PKD cells to rapamycin. Furthermore, combinations of mTOR and PLD inhibitors have a synergistic effect in reducing cell proliferation and mTOR pathway activation in PKD cells.

Discussion

PKD1/PKD2 gene mutations causes aberrant signaling through the PC1-tuberin-mTOR complex leading to: 1) deregulated tuberin-mediated nutrient signaling [34]; 2) the vasopressin receptor 2-mediated accumulation of cyclic adenosine monophosphate (cAMP) [35]; and 3) abnormal activation of other pro-proliferative signaling pathways including Ras/Raf/ERK, cyclin-depedent kinases (Cdks), and JAK/STAT [36]. While there is a considerable amount of data supporting the hypothesis that the mTOR signaling pathway plays an important role in PKD, relatively little is known about the impact of PLD signaling on PKD [3,37]. Here we show for the first time that elevated PLD activity partly modulates the mTOR signaling pathway in PKD cells suggesting a biological role for PLD/PA in PKD progression.

In our study, we found mildly but significant elevated PLD activity in PKD cells compare with control cells. Blockade of PLD activity by either specific PLD inhibitors, honokiol or “alcohol trap” treatment lead to a reduction of PA levels and mTOR activity (Figures **2** and **4**, Figure **S1**) and in turn, profoundly decreased cell viability and proliferation and increased autophagosome formation. Firstly, PA is required for the association of mTORC1 with Raptor and mTORC2 with Rictor. PA activates mTOR complexes by interacting with the FKBP12-rapamycin binding (FRB) domain, the target of the mTOR inhibitor rapamycin [11]. Secondly, PA was found to specifically bind to and activate S6K, the downstream effector of mTORC1 by increasing phosphorylation in T389 and T421/S424 as well as S6K natural substrate protein S6 in 235/236 [38]. Furthermore, other proteins that are recruited or activated by the PLD product PA, for example PI4P5 kinase, PDK, Raf, Rac1 indicate that PLD/PA modulates survival signaling by mTOR but also by mTOR-independent signaling pathways [39]. To further elucidate the function of PLD/PA associated modulation of the mTOR pathway, we increased PA levels by adding exogenous PA to stimulate mTOR signaling. Phosphorylation of mTOR, p70S6K and Akt increased in a time-dependent manner, suggesting that PA activates both mTORC1 and mTORC2 pathways at multiple levels. Of note, exogenous PA reversed the anti-proliferative effect of PLD inhibitors, indicating that the effects of PLD inhibitors were governed not exclusively via mTOR signaling. These observations suggest that PLD/PA play an important role in modulating mTOR signaling pathway in PKD.

PLD activity in mammalian cells is mediated by two different isoforms, PLD1 and PLD2, which exhibit different regulation and subcellular locations [40,41]. The relative contribution of the two isoforms in mTOR pathway is not entirely clear but it appears that depending on the particular system, either PLD isoform can sustain mTOR pathway activation. In the present study, we used isoform-selective PLD inhibitors which have been reported to inhibit the respective isoforms with > 100-fold selectivity both in *in vitro* assays and in cells [26]. We confirmed the PLD isoform specific blockage of our applied inhibitors in human PKD cells. We observed that in PKD cells, mTOR activation was more sensitive to PLD1 than to PLD2 inhibition. The PLD1 inhibitor reduced more efficiently cell proliferation than the PLD2 inhibitor, which correlated with the sensitivity of the mTOR pathway activation to PLD isoform inhibition. The results have to be interpreted in the context that the dose-dependent effects of the PLD inhibitors only provide limited information regarding the role of these PLD isoforms in the mTOR pathway. It will therefore be important to investigate the subcellular localization of PLD isoforms and mTOR in PKD,

as well as the effects of modulating the expression of different PLD isoforms on mTOR signaling in PKD cells in future studies. It should be noted that PLD inhibitors had anti-proliferative effects on both PKD and normal renal tubular epithelial cells.

Autophagy describes the process by which cytoplasmic materials including damaged or aged organelles and long-lived proteins reach the lysosomes for degradation and recycling by lysosomes [15]. Autophagy plays an important role in various aspects of cell physiology, especially cell survival during nutrient or energy limitation. However, autophagy can also trigger cell death and impair cellular functions in other contexts, underscoring its nature as a double-edged sword that can be either protective or injurious depending on the cellular environment, the nature and intensity of the stimulus, and the levels of autophagy [16,42]. There is so far only one published study reporting increased LC3-II conversion in homozygous Han: SPRD rat kidneys (Cy/Cy) at an advanced stage of PKD, indicating autophagy deregulation in PKD [18]. Aberrant signaling through the mTOR pathway is a common feature of PKD and there is a crosstalk between mTOR and autophagy in many types of cells [17,43]. In our study, mTOR inhibitors did not induce autophagy in PKD cells, even at high concentrations, suggesting that PKD cells are resistant to rapamycin-induced autophagy. Interestingly, we found that PLD inhibitors and honokiol treated PKD cells displayed autophagy suggesting that PLD-induced-autophagy bypassed the mTOR signaling pathway. In line with our findings, Takita T et al. reported that diacylglycerol kinase inhibitor reduced PA level induced autophagy in neuronal cells [44], whereas, Dall' Armi et al. reported that PLD1 modulated autophagy via association with the endosomal system, partially re-localizing to the outer membrane of autophagosome-like structures [45]. Thus, PLD modulates autophagy in different manners, depending on cell type and subcellular localization: On one hand, PLD/PA interact with mTOR, PI3K-Ras/ERK signaling pathways, both regulate negatively autophagy. On the other hand, PLD1 has been shown to foster autophagy by co-localized with LC3 during starvation. Such a dual role has been previously described for Vps34, a lipid enzyme that is required for autophagy, yet also stimulates mTOR [46,47].

Rapamycin-based therapeutics effectively decreased cyst growth and preserved renal function in a variety of animal models for PKD [28,29,48]. However, conflicting results were obtained in clinical trials. Two clinical trials did not show a beneficial effect in ADPKD patients, both in early and more progressive disease stages [31,32]. One of the possible factors confounding the interpretation of these results could be the dose of mTOR inhibitors used. The levels of rapamycin tolerated in humans are lower than in mice [28]. Based on our results, elevated activation of PLD may mitigate the effect of rapamycin on human PKD cells. Indeed, combining mTOR and PLD inhibitors enhanced the rapamycin-sensitivity of PKD cells. Therefore, combination therapies that include rapamycin and strategies that suppress PLD activity could be used to target mTOR signaling in PKD.

In summary, our data shows that elevated PLD activity in PKD cells. mTOR signaling pathway was partly modulated in a PLD/PA-dependent way (Figure **7**). Targeting PLD blocked cell proliferation, decreased mTOR signaling and induced autophagy formation. Combination of mTOR and PLD inhibitors has a synergistic effect on retarding cell proliferation and blocking mTOR pathway. Targeting PLD may provide a new potential therapeutic approach for PKD.

Fig. 7

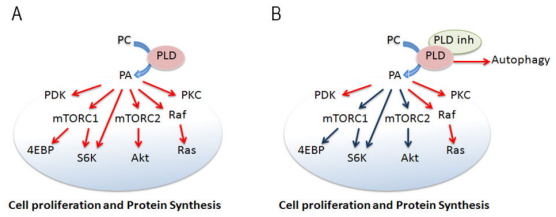

Figure 7. PLD and mTOR pathway in PKD. (A) Elevated PLD activity stimulates the mTORC1, mTORC2 and other relative pathways and promotes cell proliferation in PKD cells. (B) Inhibition of PLD reduces phosphorylation of downstream targets of mTORC1 and mTORC2, and induces autophagy in PKD cells.

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Supporting Information

Figure S1. Honokiol impaired cell growth and blocked mTOR signaling in Cy/+ cells. Effect of honokiol on (A) cell viability and DNA synthesis determined by MTS and BrdU. 48 h after treatment initiation. (B) Honokiol affected PLD/PA and mTOR signaling in a dose-dependent way. Western blots analyzing the expression of phospho-PLD1, PLD1, phospho-PLD2, PLD2, phospho-Akt, Akt, phospho-S6K, S6K, phospho-S6 and S6 either upon treatment with the indicated concentration of PLD1-/2- inhibitor for 48 h or without treatment. Blots are representative of three independent experiments. Data are expressed as mean ± SD and were analyzed by one-way ANOVA. * $p< 0.05$, ** $p< 0.01$.
(TIF)

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Author Contributions

Conceived and designed the experiments: YL AA AS. Performed the experiments: YL AK UZ. Analyzed the data: YL AS. Contributed reagents/materials/analysis tools: AO DW. Wrote the manuscript: YL AA AS.

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10 Submitted First Author Manuscript

Dual mTOR/PI3K inhibition limits pro-proliferative PI3K-dependent pathways activated upon mTOR inhibition in autosomal dominant polycystic kidney disease

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by development of renal cysts leading to renal failure in adulthood[100]. Mammalian target of rapamycin (mTOR) inhibition slows polycystic kidney disease (PKD) progression in animal models, but randomized controlled trials failed to prove efficacy of mTOR inhibitor treatment[6, 80, 101-103]. Here we demonstrate that mTOR inhibitors result in the removal of negative feedback loops and up-regulate pro-proliferative phosphatidylinositol 3-kinase (PI3K)-Akt and PI3K-extracellular signal-regulated kinase (ERK) signaling in rat and mouse polycystic kidney models. Dual mTOR/PI3K inhibition with NVP-BEZ235 abrogated these pro-proliferative signals and normalized kidney morphology and function by blocking proliferation and fibrosis. Our findings suggest that multi-target PI3K/mTOR inhibition may represent an effective treatment for ADPKD.

TEXT

Autosomal dominant polycystic kidney disease (ADPKD) is the most common potentially lethal monogenetic hereditary kidney disease. The disease is characterized by the progressive dilation of renal tubules that eventually form cysts, leading to renal failure[100]. The mammalian target of rapamycin (mTOR) kinase is a master regulator of protein synthesis and proliferation and is aberrantly activated in ADPKD[104, 105].

A dual negative feedback loop has been reported in human cancer: mTOR/S6K activation attenuates upstream phosphatidylinositol 3-kinase (PI3K) pathway activation, while treatment with mTOR inhibitors (rapamycin and its analogs) lead to a hyperactive insulin receptor substrate 1 (IRS-1)/PI3K pathway, and in turn increases the signaling toward to the pro-proliferative extracellular signal-regulated kinases (ERK) and Akt pathways[12, 13, 106, 107]. We hypothesized that mTOR inhibition might also lead to compensatory up-regulation of the PI3K-dependent pathway in ADPKD by the release of mTOR controlled negative feedback loops that might attenuate the efficacy of mTOR inhibitors.

To explore this hypothesis we examined the effect of mTOR inhibitors on these dual negative feedback loops *in vitro* and in animal model of ADPKD. First we treated Han:SPRD male rats, a well characterized strain (Cy/+) that phenotypically resembles human ADPKD, with the rapamycin analog everolimus (gavage 3mg/kg/day) from 4 to 16 weeks of age[63, 80, 108]. Everolimus treatment increased the activity of readouts of PI3K/Akt and PI3K/ERK in polycystic kidney. **Figure 1a** shows that phosphorylation of T202/204-ERK, T308-Akt and S473-Akt were increased in polycystic kidneys of Cy/+ animals whereas, in *wild type* animals, these pathways were not activated. The *in vivo* results were extended by *in vitro* studies: mTOR inhibition by everolimus caused Akt and ERK activation while decreasing the activity of mTOR readouts (phosphorylation of T389-S6K, T421/S424-S6K, S235/236-S6 and S240/244-S6) in cultured Han:SPRD Cy/+ male renal tubular epithelial cells (TEC) in a dose-dependent manner **(Fig. 1b)**. Our *in*

vitro and animal data indicate that mTOR inhibitors cause the activation of pro-proliferative signaling pathways in polycystic kidneys.

Furthermore, we assessed these signaling pathways in ADPKD patients enrolled in the SUISSE ADPKD study[6]. While polycystic kidney specimens were not available from this trial, peripheral blood mononuclear cells (PBMCs) were isolated from patients before and after treatment with either sirolimus or standard care for 6 months[109-111]. The phosphorylation of ribosomal S6 protein was blocked whereas ERK phosphorylation was markedly increased among sirolimus treated patients (n=3) and phospho-Akt was increased in 2 of 3 sirolimus treated patients **(Supplementary Fig. 1).** The mTOR signaling pathway activity status in PBMC correlated imperfectly with the one in the polycystic kidney. Therefore, we further investigated the impact of activation of the mTOR signaling upon treatment with mTOR inhibitors in Han:SPRD Cy/+ male renal tubular epithelial cells[111]. Triple inhibition of mTOR, ERK and Akt by using everolimus, UO126, and perifosine as corresponding inhibitors, caused considerably more effective loss of cell viability and inhibition of DNA synthesis than any double drug combination **(Fig. 1c)**. Western blot analysis confirmed the effect of each inhibitor on the respective pathway **(Fig. 1d).** We then applied NVP-BEZ235, a dual mTOR/PI3K inhibitor, with proven efficacy in various human cancer models[112-114]. We evaluated the ability of NVP-BEZ235 treatment to halt PKD progression *in vivo:* we administrated low-dose (15 mg/kg/day) and high-dose (50 mg/kg/day) NVP-BEZ235 to Han:SPRD male rats between 4 and 9 weeks of age **(Fig. 2a)**. This treatment, in particular at the higher dose regimen, had a dramatically positive effect on all aspects reflecting PKD disease burden: the renal function (blood urea nitrogen, serum creatinine concentration, and urine albumin/creatinine ratio) was altogether comparable to that in *wild type* animals **(Fig. 2d-f and Supplementary Table 1, 2).** In addition, the kidney morphology (evaluated as two kidneys weight/total body weight ratio, cyst volume, parenchymal fibrosis, and cell proliferation) was similar in NVP-BEZ235-treated PKD and in *wild type* animals **(Fig. 2b,c Fig. 3a-d and Supplementary Table 1, 2)**. NVP-BEZ235 was generally well tolerated with no mortality reported. Notably, the renal function after 5 weeks of treatment was better in NVP-BEZ235-treated compared to everolimus-treated animals **(Supplementary Table 3)**[80].

We then studied the effect of NVP-BEZ235 treatment on mTOR and its dual feedback loop pathway *in vivo*, in order to describe a possible mechanism of action of this compound for treatment of PKD. NVP-BEZ235 blocked the mTOR pathway, as shown by decrease of phospho-T421/S424-S6K and phospho-S235/236-S6, i.e. both accepted as readout of mTOR pathway activity. Expression of phospho-T308-Akt, phospho-S473-Akt and phospho-T202/204-ERK, used to monitor activation of the dual feedback loop, was prevented by the high-dose regimen, whereas the low-dose regimen was already sufficient to reduce the levels of phospho-S473-Akt and phospho-T202/204-ERK **(Fig. 3e and Supplementary Fig. 3a)**. Kidney tissue sections of untreated-Cy/+ rats showed that cysts-lining epithelial cells stained strongly positive for phospho-T202/204-ERK and phospho-S235/236-S6 in the cortex and in the corticomedullary region, whereas *wild type* and NVP-BEZ235-treated animals displayed only a mild and scattered positive staining **(Fig. 3a)**. In spite NVP-BEZ235 activates ERK in some cancer cells, this effect was not evident in the polycystic kidney models tested, nor in primary renal tubular epithelial cells **(Fig. 3e and Supplementary Fig. 2b)**[112, 115, 116]. Thus, dual mTOR/PI3K inhibition abrogates the up-regulation of Akt and ERK signaling pathways, otherwise observed in the presence of single agent mTOR inhibition. Of note, NVP-BEZ235 had no effect on either molecular pathway, when analyzed in *wild type* treated kidneys, suggesting that the mode of action is specific for ADPKD **(Fig. 3e)**.

To further assess the relevance of dual mTOR and PI3K-dependent pathway inhibition as a therapy for PKD, we compared the efficacy of NVP-BEZ235 and sirolimus in an orthologous ADPKD mouse model with a conditionally inactivated *Pkd1* gene[117]. Cystogenesis was induced by tamoxifen intraperitoneal (ip) injection into pups at postnatal day 11, an animal model that is characterized by progressive renal functional decline over 4 to 6 weeks[76]. We initiated the NVP-BEZ235 treatment at an early disease state (dose before weaning 6 mg/kg, after weaning 9 mg/kg) and sirolimus (3 mg/kg) between day 12 and day 35, and at late disease state with NVP-BEZ235 (9 mg/kg) between day 21 and day 35 by ip injection (**Fig. 4a**). Early NVP-BEZ235 treatment initiation slowed renal functional loss **(Fig. 4d, e)**, lowered kidney volume **(Fig. 4b, c)**, inhibited cystogenesis **(Fig. 4f)**, fibrogenesis **(Fig.**

4g) and proliferation **(Fig. 4h)** compared with vehicle-treated group. NVP-BEZ235 inhibited mTOR targets (phospho-S6K, phospho-S6 and phospho-4EBP) and prevented the up-regulation of phospho-ERK and phospho-Akt, whereas sirolimus triggered Akt activation (**Fig. 4i and Supplementary Fig. 3**). On the other end, late NVP-BEZ235-treated mice showed high activation of phospho-T202/204-ERK, phospho-T421/S424-S6K, and phospho-S235/236-S6, concomitant to the relative renal function and morphology, thus illustrating that the therapeutic effect is due to inhibition of these pathways (**Supplementary Fig. 4 and Supplementary Table 4, 5**). In line with this, NVP-BEZ235 treatment retarded cystogenesis, preserved renal function and blocked proliferation more efficiently than sirolimus **(Fig. 4b-h)**. Our study therefore suggests that NVP-BEZ235 inhibits both the mTOR and the PI3K/Akt pathway, in turn decreasing signaling through the PI3K/ERK pathway (**Fig. 4j**). The NVP-BEZ235 was well tolerated and body weight gain was higher in mice treated with NVP-BEZ235 compared to sirolimus-treated mice (**Supplementary Table 4**). Notably, in male PCK rats, an established autosomal recessive polycystic kidney (ARPKD) model, NVP-BEZ235 did not improve and sirolimus even worsened the renal function, suggesting that NVP-BEZ235's beneficial action is specific for ADPKD **(Supplementary Fig. 5 and Supplementary Table 7)**[68, 118].

In conclusion, blockade of the mTOR pathway by using mTOR inhibitors triggers up-regulation of pro-proliferative PI3K-dependent pathways, either through the PI3K/Akt or PI3K/ERK pathway in ADPKD. Dual mTOR/PI3K inhibition specifically prevents the activation of these feedback loops and, when applied in early stage disease, this treatment normalizes the renal function and the morphology of ADPKD rat and mice models **(Fig. 4j)**.

METHODS

Experimental animals and study design

Han:SPRD rat

The Han:SPRD rat colony was established in our animal facility from a litter which was obtained from the Rat Resource and Research Center (Columbia, MO). Heterozygous cystic (Cy/+) and wild-type (+/+) male rats were used in this study. Figure 2a display the rat study set up: we applied by gavage NVP-BEZ235 (Novartis) at low-dose (15mg/kg/day), high-dose (50mg/kg/day) and vehicle solution (90% PEG300 and 10% 1-mehtyl-2-pyrrolidinone, Sigma-Aldrich) from 4 to 9 weeks of age. Blood was collected at 4, 6.5 and 9 weeks of age for determination of serum creatinine and BUN levels. 24 h urine samples were collected in metabolic cages one day before the rats were killed. We applied by gavage everolimus (E, 3mg/kg/day) and vehicle solution (V) from 4 to 16 weeks of age. Rats were anesthetized with isoflurane and kidneys were harvested for further analysis.

Pkd1 conditional knockout mouse

Professor G.G Germino supplied the Pkd1 conditional knockout mouse. We induced Cre recombinase activity by intraperitoneal injection of tamoxifen (1.25 mg/10g, dissolved in corn oil (Sigma-Aldrich) at day 11 of age into pups. Figure 4a display the mice study set up: The mice were randomly allocated to seven groups; $Pkd1^{cond/cond}$, Cre^{+} vehicle (V+), $Pkd1^{cond/cond}$, Cre^{+} early-phase NVP-BEZ235 (N+), $Pkd1^{cond/cond}$, Cre^{+} late-phase NVP-BEZ235 (LN+), $Pkd1^{cond/cond}$, Cre^{+} early-phase sirolimus (S+), $Pkd1^{cond/cond}$, Cre^{-} vehicle (V-), $Pkd1^{cond/cond}$, Cre^{-} early-phase NVP-BEZ235 (N-), and $Pkd1^{cond/cond}$, Cre^{-} early-phase sirolimus (S-). For the N+ and N- groups, animals were treated with 6 mg/kg/day NVP-BEZ23 from day 12 to day 20, and later with 9 mg/kg/day until day 35. For the S+ and S- groups, 3mg/kg/day sirolimus was administrated from day 12 to day 35. For the LN+ group, mice were treated with 9mg/kg/day NVP-BEZ235 from day 21 to day 35. NVP-BEZ235, sirolimus and vehicle solutions were administrated by intraperitoneal injections. 24 h urine samples were collected by metabolic cage at day 35. Mice were anesthetized with isoflurane at day 36, and blood was obtained by cardiac

puncture for determination of serum creatinine and BUN levels and kidneys were harvested for further analysis.

Biochemical analysis

Rat and mice serum creatinine and BUN levels were detected with UniCel® DxC 800 Synchron® Clinical (Zurich Integrative Rodent Physiology). The GenWay Albumin Elisa Kits for rat were used for rat urine albumin concentration measurement, according to the manufacturer's protocol.

Cells

Primary renal tubular epithelial cells

Heterozygous cystic (Cy/+) and wild-type normal (+/+) male rats, aged 8-weeks were used in this study. Primary renal tubular epithelial cells (TEC) were isolated as follows: kidneys were minced and digested by 1 mg/ml collagenase with gentle agitation for 1 hour at 37°C. The suspension was allowed to sediment for 1 minute twice. Cells were collected by harvesting the supernatant and then washed 3 times with 10% FBS/HBSS. Isolated cells were re-suspended in K1 medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% FBS,10 mM HEPES, 42 mM sodium bicarbonate, 50 ng/ml insulin, 50 nM hydrocortisone, 50 ng/ml transferrin, 5 pM trijodothyronine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were seeded in collagen type 1-precoated culture dishes for further study.

Human peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from ADPKD patients by BD Vacutainer® CPT™ Cell Preparation Tube, according to the manufacturer's protocol.

Cell viability and proliferation assays

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based CellTiter 96 AQueous One assay (Promega) was used to quantify cell viability. Cell proliferation was assessed using BrdU Cell Proliferation ELISA Kit (Roche Applied Science), which quantifies cell proliferation by measuring DNA synthesis. The assay was

performed according to the manufacturer's instructions and results were expressed as mean absorbance of the samples measured in an ELISA plate reader.

Western blot analysis

Kidneys were homogenized with ice-cold lysis buffer containing 40 mM Hepes, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM potassium pyrophosphate, 10 mM glycerol phosphate, 50 mM NaF, 0.5 mM $NaVO_3$, 1% Triton-X 100, and protease inhibitor mixture (pH 7.6). Total cell lysate were prepared in the same ice-cold lysis buffer. Lysates were cleared by centrifugation. The amount of protein was determined by using the BCA Protein Assay (Thermo Scientific). Equal amounts of lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and probed with antibodies against phospho-ERK (Thr202/Tyr204), ERK, phospho-p70 S6K (Thr421/Ser424 and Thr389), p70 S6K, phospho-Akt (Ser473 and Thr308), phospho-mTOR (Ser2448), mTOR, phospho-S6 ribosomal protein (Ser235/236 and Ser240/244), S6 ribosomal protein, phospho-4E-BP1 (Thr37/46), 4E-BP1 (all from Cell Signaling Technology), Akt (Abcam), GAPDH (Merck Millipore), β-Tubulin (Sigma-Aldrich). Sheep anti-mouse IgG-HRP and donkey anti-rabbit IgG-HRP were used as secondary antibodies (GE Healthcare). Quantification of phosphorylated protein was normalized for either relative total protein or loading control protein using a densitometer.

Histomorphometric analysis and immunohistology

Paraffin-embedded kidney section (4 µm) including cortex, medulla and papilla were stained with PAS to measure cyst volumes and with Sfog staining and Masson trichrome staining to measure fibrosis. Images were analyzed by HistoQuest image analysis software (TissueGnostics) to quantify total, cystic and fibrotic area. For immunohistochemical analyses, sections were processed for heat-mediated antigen retrieval in citrate buffer (pH 6.0) (Vector Laboratories, Inc), and anti-phospho-S6 ribosomal protein (Ser235/236), anti-phospho-ERK (Thr202/Tyr204) and Ki-67 (all from Cell Signaling Technology)

were used. The proliferation index was calculated as percentage of Ki-67 positive nuclei to the total nuclei per cyst.

Statistical analysis

Data are presented as mean ± SD. Statistical differences between treatment groups were performed by the unpaired two-tailed *t*-test using GraphPad Prism version 4.0 (GraphPad). $P < 0.05$ was considered to be statistically significant.

FIGURE LEGENDS

Figure 1 Mammalian TOR pathway blockade triggered dual PI3K-depedent feedback loops stemming from S6K to PI3K/ERK and to PI3K/Akt in PKD. (a) Han:SPRD rats whole kidney lysates western blot analysis and densitometry of PI3K/Akt and PI3K/ERK pathway readouts after 12 weeks everolimus (E) or vehicle (V) treatment (n=4 per group). ** $p < 0.01$ indicated by brackets . (b) Western blots analyzing the activation of readouts of PI3K/Akt and PI3K/ERK (right panel). mTOR (left panel) upon treatment with indicated everolimus concentration for 48 h. (c) Cell viability (MTS, left panel) and DNA synthesis (BrdU, right panel) of Cy/+ renal primary tubular epithelial cells (TEC) after combined application of mTOR (everolimus, E), Akt (UO126, U) and ERK (perifosine, P) inhibitors for 48 h. # $p < 0.001$ compare to control, except where indicated by brackets. (d) Western blot analysis of mTOR, PI3K/Akt and PI3K/ERK pathways after 48 h combination treatment with the indicated inhibitors in Cy/+ TEC. Graphs are representative of three independent experiments.

Figure 2 Efficacy of NVP-BEZ235 treatment on Han:SPRD rats renal function. (a) Study design scheme. Dose-dependent effect of NVP-BEZ235 on PKD progression. (b) Representative PAS stained kidney sections of NVP-BEZ235-treated and vehicle-treated Han:SPRD rats. Quantitative analysis of (c) two kidney to total body weight ratio (2K/TBW), (d) 24 h urine albumin to creatinine ratio, (e) BUN, (f) serum creatinine in Han:SPRD rats are displayed. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ indicated by brackets (mean ± SD, ANOVA). The number of rat per group is indicated in **Supplementary Tables 1** and **2**.

Figure 3 Impact of NVP-BEZ235 treatment on renal morphology and molecular pathways. (a) Representative kidney sections of NVP-BEZ235-treated and vehicle-treated rats. Shown are renal histological and immunohistochemical staining with Masson trichrome staining, Ki-67, p-ERK (T202/204) and p-S6 (S240/244) from top to bottom panels, respectively.

Quantitative analysis of (b) cystic index, (c) fibrosis index and (d) proliferation index in Han:SPRD rats are displayed. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ indicated by brackets (mean ± SD, ANOVA). The number of rat per group is indicated in **Supplementary Tables 1** and **2**. (e) Western blot analysis of Han:SPRD whole kidney lysates for readouts of mTOR, PI3K/Akt and PI3K/ERK pathways after 5 weeks treatment with NVP-BEZ235-treated and vehicle-treated rat. Representative results of three independent experiments are shown.

Figure 4 Efficacy of NVP-BEZ235 and sirolimus treatment in *Pkd1* conditional knockout mice. (a) Study design scheme. (b) Representative PAS stained kidney sections of NVP-BEZ235-treated, sirolimus-treated and vehicle-treated mice. Quantitative analysis of (c) two kidneys to total body weight ratio (2K/TBW), (d) BUN, (e) serum creatinine, (f) cystic index, (g) fibrosis index and (h) proliferation index are shown. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ indicated by brackets (mean ± SD, ANOVA). The number of mice per group is indicated in **Supplementary Tables 3** and **4**. (i) Whole kidney lysates Western blot analysis of mTOR, PI3K/Akt and PI3K/ERK pathways (j) Schematic representation of the pathways examined in the study. mTOR pathway is abnormally activated in polycystic kidneys (left panel). Treatment with mTOR inhibitor results in a hyperactive PI3K-dependent pathway, increasing the signal toward the PI3K/Akt and PI3K/ERK pathway (middle panel). Dual mTOR/PI3K inhibitor NVP-BEZ235 may provide a therapeutic benefit for ADPKD by abrogating the activation of PI3K/Akt and PI3K/ERK pathways and in turn block the mTOR pathway (right panel). Red denotes activated; blue, inhibited.

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AUTHOR CONTRIBUTIONS

Y.L. designed and performed the experiments, analyzed data and wrote the manuscript. X.W. and X.F. performed the *in vivo* experiments with NVP-BEZ235 treatment. D.C. and A.A. designed the studies and discussed results. T.J.W. supplied the *Pkd1* conditional knockout mice. A.L.S. designed the experiments and studies, supervised the work and wrote the manuscript.

COMPETEING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Figure 1

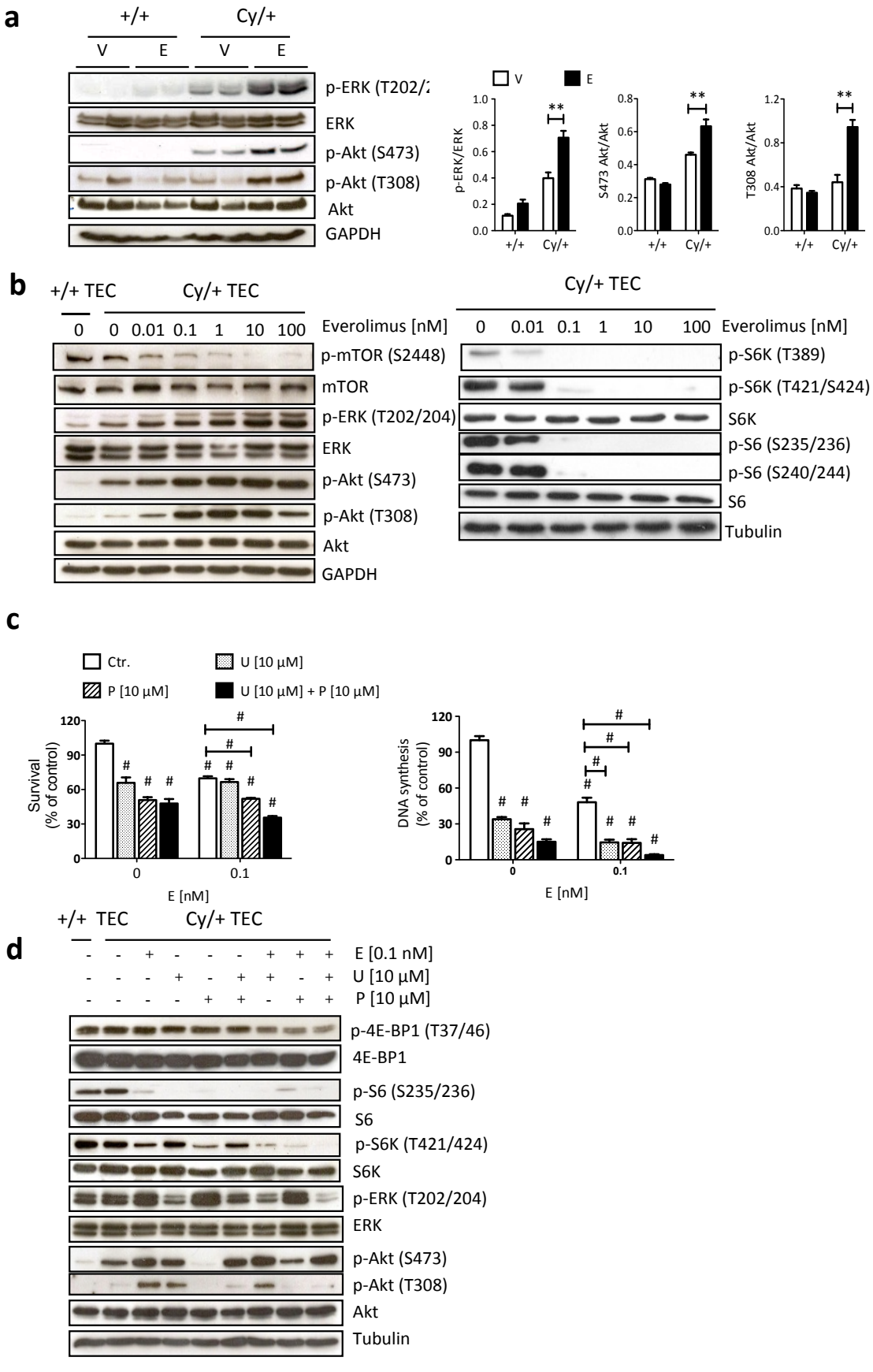
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Figure 2

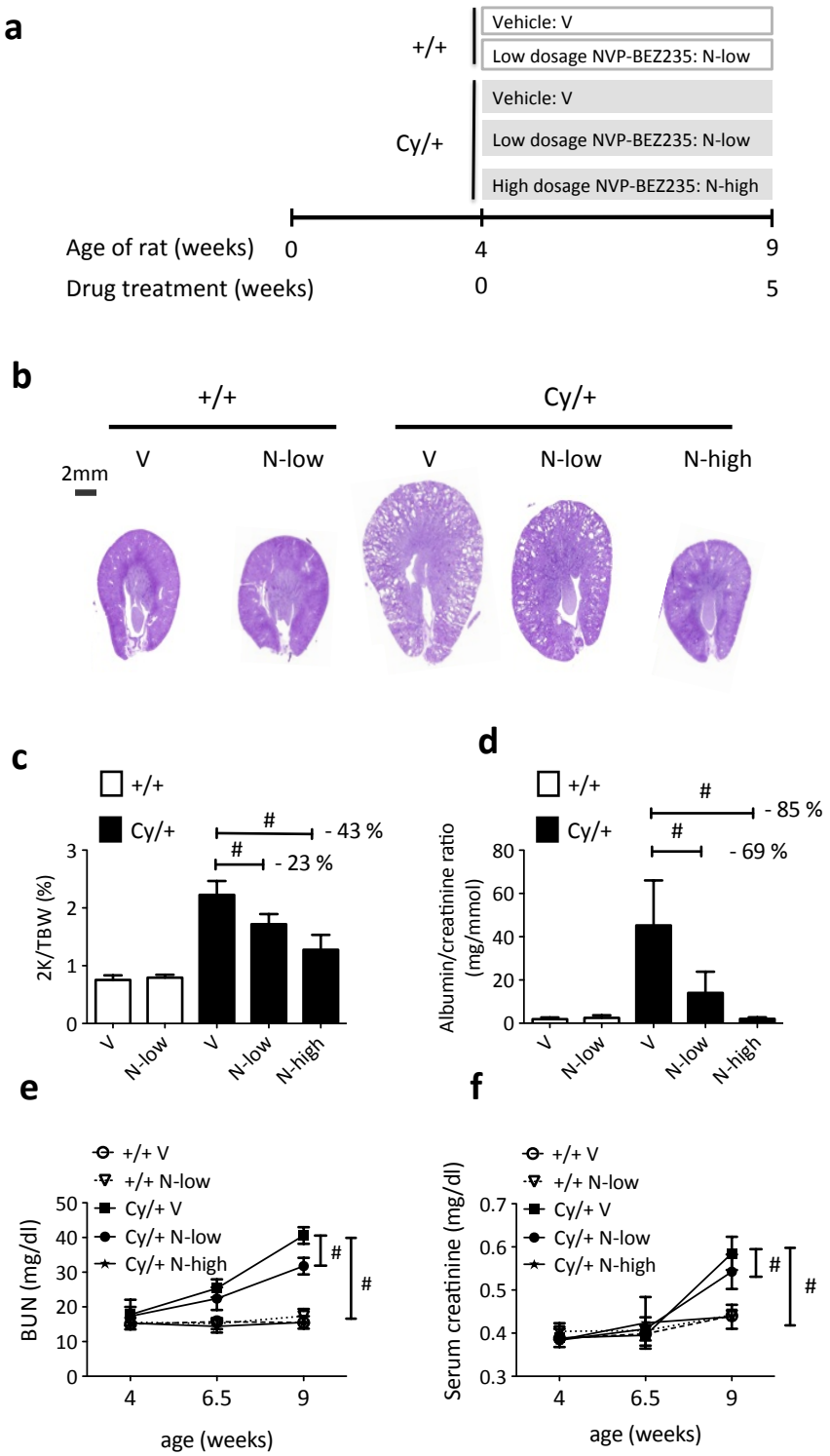
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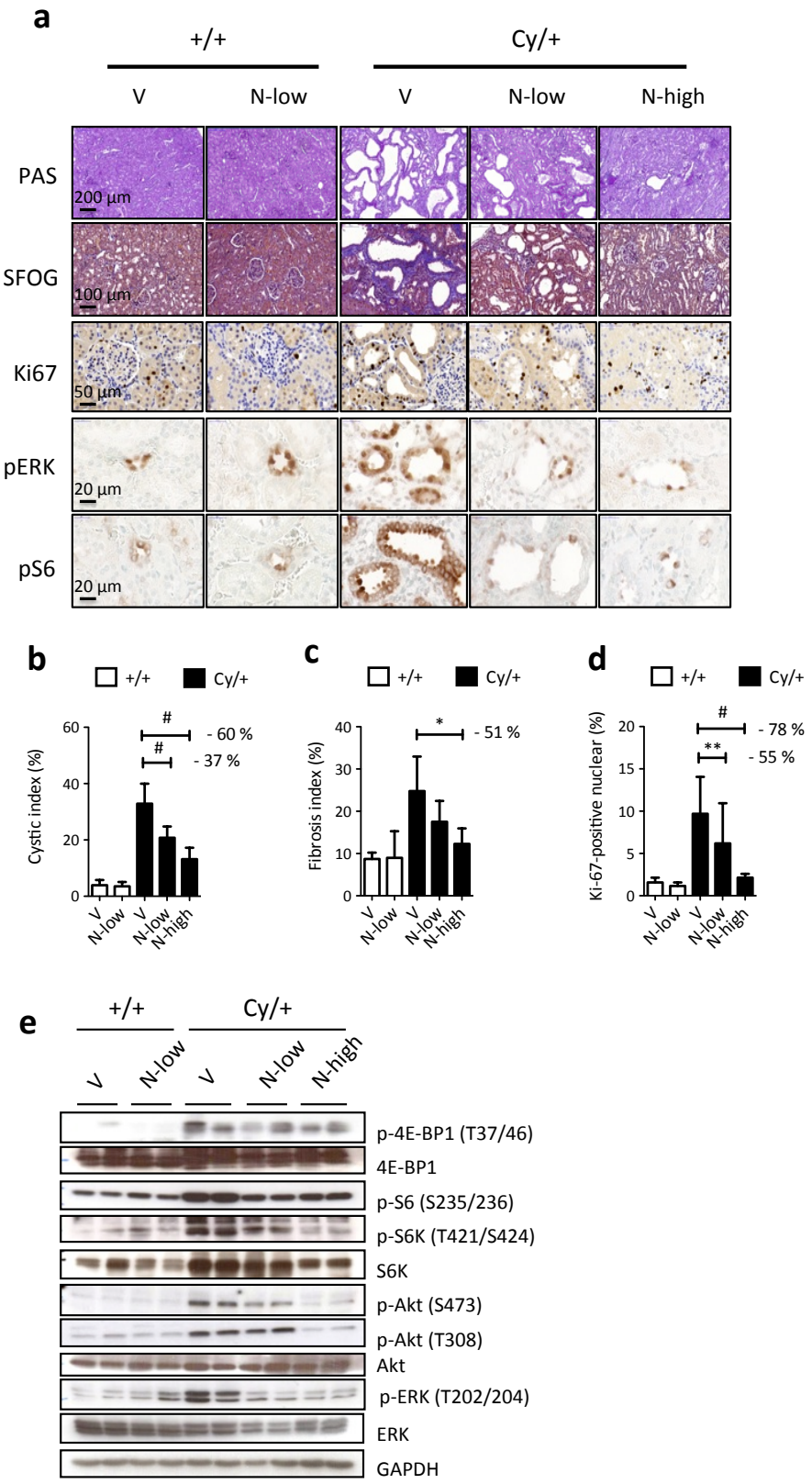
Figure 3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- 37 %
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Fibrosis index (%)
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Ki-67-positive nuclear (%)
- 78 %
- 55 %
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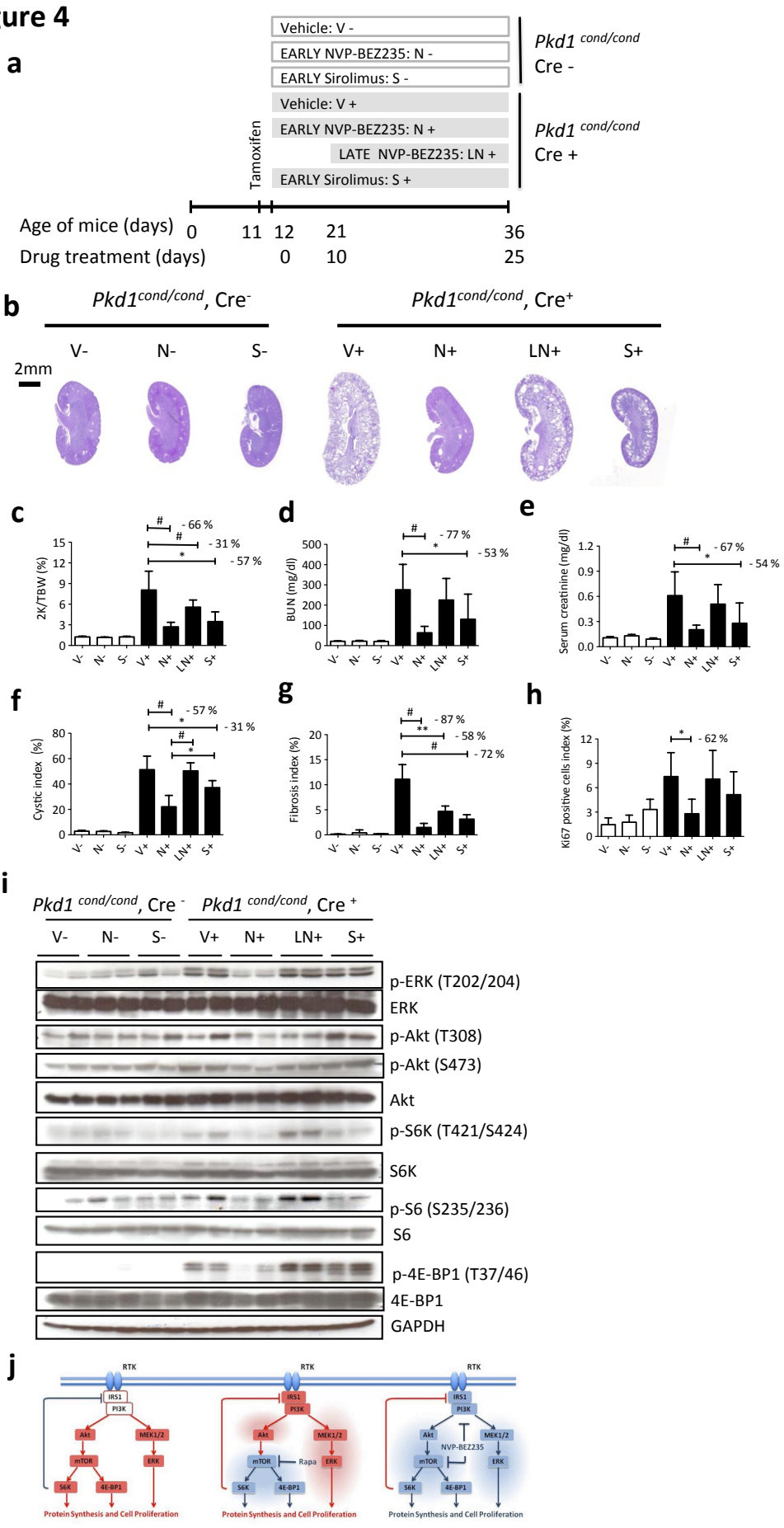
p-S6 (S235/236)
p-S6K (T421/S424)
S6K
p-Akt (S473)
p-Akt (T308)
Akt
p-ERK (T202/204)
ERK
GAPDH

Figure 4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Vehicle: V -
EARLY NVP-BEZ235: N -
EARLY Sirolimus: S -
Pkd1 cond/cond
Cre -
Vehicle: V +
EARLY NVP-BEZ235: N +
LATE NVP-BEZ235: LN +
EARLY Sirolimus: S +
Tamoxifen
Pkd1 cond/cond
Cre +
Age of mice (days) 0 11 12 21 36
Drug treatment (days) 0 10 25
b
Pkd1cond/cond, Cre-
Pkd1cond/cond, Cre+
V- N- S- V+ N+ LN+ S+
2mm
c
2K/TBW (%)
- 66 %
- 31 %
- 57 %
d
BUN (mg/dl)
- 77 %
- 53 %
e
Serum creatinine (mg/dl)
- 67 %
- 54 %
f
Cystic index (%)
- 57 %
- 31 %
g
Fibrosis index (%)
- 87 %
- 58 %
- 72 %
h
Ki67 positive cells index (%)
- 62 %
i
Pkd1 cond/cond, Cre -
Pkd1 cond/cond, Cre +
V- N- S- V+ N+ LN+ S+
p-ERK (T202/204)
ERK
p-Akt (T308)
p-Akt (S473)
Akt
p-S6K (T421/S424)
S6K
p-S6 (S235/236)
S6
p-4E-BP1 (T37/46)
4E-BP1
GAPDH
j
RTK
IRS1
PI3K
Akt
MEK1/2
mTOR
ERK
S6K
4E-BP1
Rapa
NVP-BEZ235
Protein Synthesis and Cell Proliferation

SUPPLEMENTARY METHODS

Experimental animal and study design

PCK rat

The PCK rat colony was established in our animal facility from a litter that was obtained from Charles River Laboratories. Male rats were used in this study. We applied by gavage NVP-BEZ235 (N) at 50mg/kg/day, sirolimus (S) at 3mg/kg/day and vehicle solution (V) from 3 to 6 weeks of age. Blood was collected at 6 weeks of age for determination of serum creatinine and BUN levels and 24 h urine samples were collected in metabolic cages one day before the rats were killed. Rats were anesthetized with isoflurane and kidneys were harvested for further analysis.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 Blocking the mTOR pathway by sirolimus triggered the activation of ERK and Akt in PBMCs of ADPKD patients. Immunoblots and densitometry readouts of mTOR, PI3K/Akt and PI3K/ERK pathways in PBMCs protein lysates from autosomal dominant polycystic kidney disease (ADPKD) patients allocated to sirolimus (Sir, N=3) and standard care (Placebo, N=2) before (B) and after (A) treatment (interval time = 6 months). * $p < 0.05$, ** $p < 0.01$ (mean ± SD, ANOVA) indicated by brackets.

Figure S2 Effect of NVP-BEZ235 on molecular pathways *in vitro*. Western blot analysis of mTOR pathway, PI3K/Akt and PI3K/ERK pathways treated for 48 h by various concentrations [nM] of NVP-BEZ235 in Cy/+ TEC. Data are representative of three independent experiments.

Figure S3 Quantitative analysis of immunoblots of mTOR, PI3K/Akt and PI3K/ERK pathways *in vivo*. Densitometry readouts of mTOR, PI3K/Akt and PI3K/ERK pathways in kidney tissue lysates from (a) Pkd1 conditional knock out mice and (b) Han:SPRD rats. (n= 4 per group). * $p < 0.05$, ** $p < 0.01$, #

p< 0.001 indicated by brackets (mean ± SD, ANOVA). Data are representative of three independent experiments.

Figure S4 Kidney histology and immunohistochemical staining in Pkd1 conditional knock out mice. Representative kidney sections from NVP-BEZ235-, sirolimus- and vehicle-treated mice. Kidney histology and immunohistochemical staining with PAS, Masson trichrome, Ki-67, p-ERK (T202/204) and p-S6 (S240/244) are displayed from top to bottom panels.

Figure S5 NVP-BEZ235 and sirolimus treatment efficacy in PCK rats. (a) Study design scheme. (b) Representative PAS stained and Masson trichrome stained kidney sections of NVP-BEZ235-treated (N), sirolimus-treated (S) and vehicle-treated (V) mice. Quantitative analysis of (c) two kidneys to total body weight ratio (2K/TBW), (d) BUN, (e) serum creatinine, (f) cystic index and (g) fibrosis index are shown. * $p < 0.05$, ** $p < 0.01$, # $p < 0.001$ indicated by brackets (mean ± SD, ANOVA). The number of mice per group is indicated in Supplementary Tables 7**.** (h) Whole kidney lysates Western blot analysis of mTOR, PI3K/Akt and PI3K/ERK pathways.

Figure S1

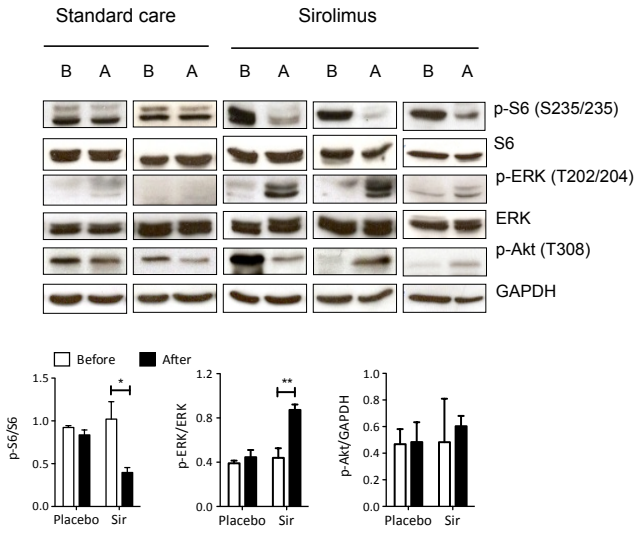


Figure S2

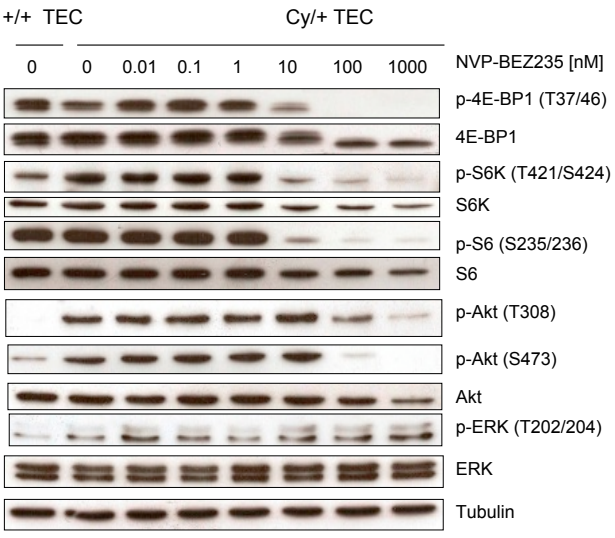


Figure S3

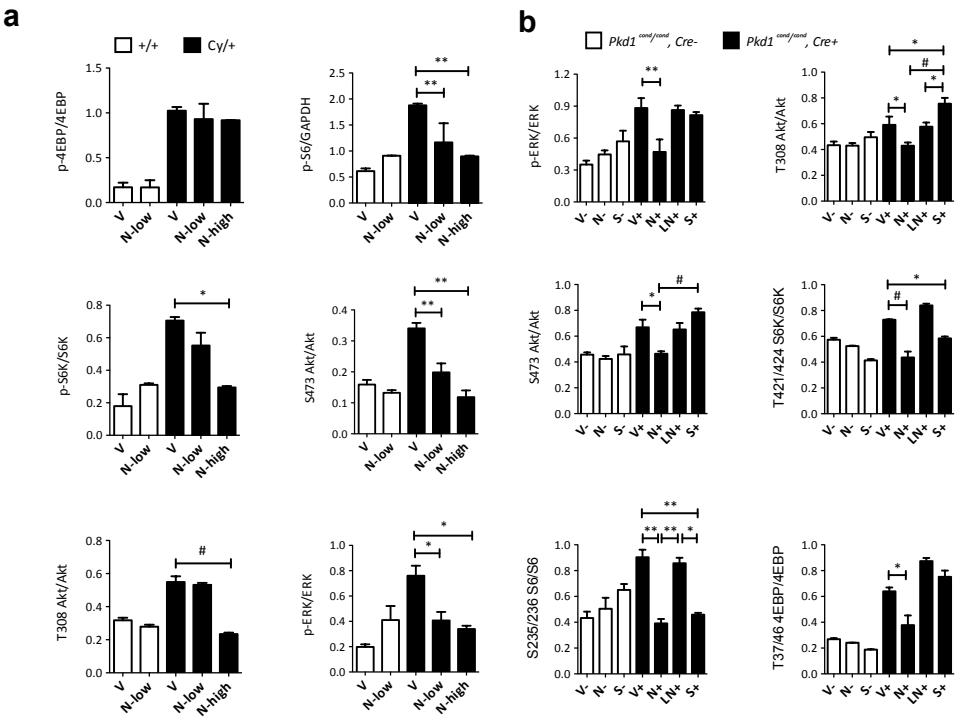


Figure S4

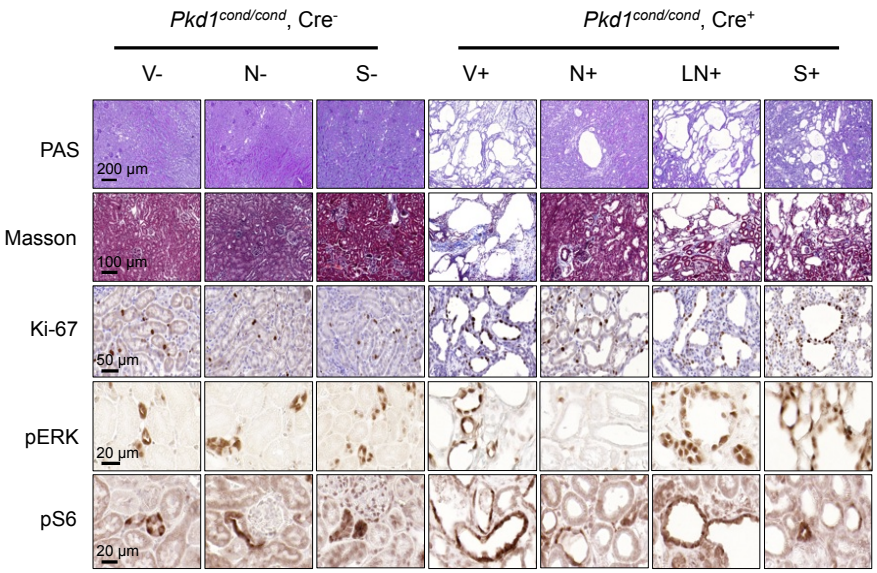
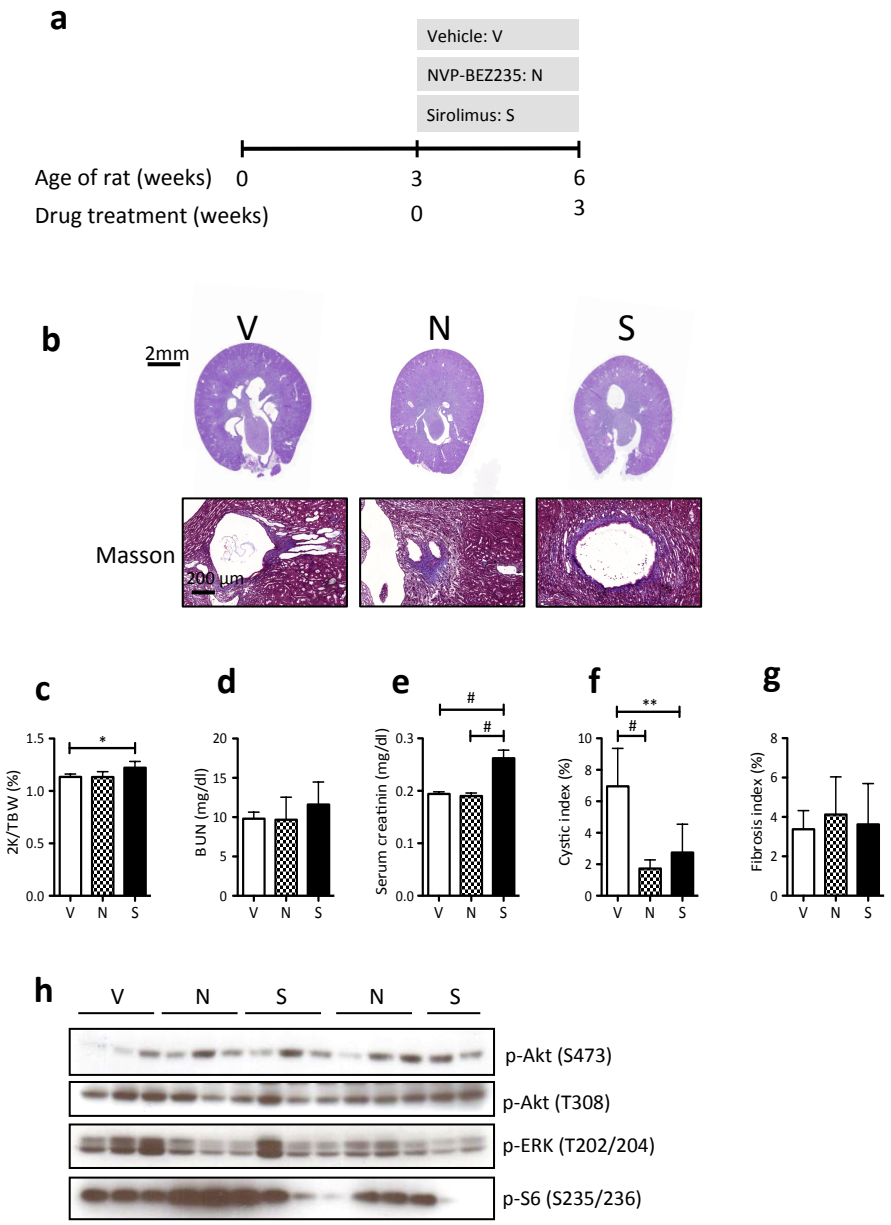


Figure S5


a
Vehicle: V
NVP-BEZ235: N
Sirolimus: S
Age of rat (weeks) 0 3 6
Drug treatment (weeks) 0 3
b
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Cystic index (%)
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Fibrosis index (%)
h
V N S N S
p-Akt (S473)
p-Akt (T308)
p-ERK (T202/204)
p-S6 (S235/236)

Supplementary Tables

Table S1 NVP-BEZ235 effect on renal function in male Han:SPRD rats

	Time Point (weeks)	*+/+ Vehicle* n=5	*+/+ NVP-low* n=7	*Cy/+ Vehicle* n=8	*Cy/+ NVP-low* n=8	*Cy/+ NVP-high* n=5
2K/TBW (%)	9w	0.75 ± 0.08	0.79 ± 0.05	2.23 ± 0.24	1.72 ± 0.18***	1.28 ± 0.26***
TBW (g)	4w	90.6 ± 18.74	102.14 ± 22.23	115.5 ± 13.2	116.13 ± 10.88	91 ± 4.24
	6.5w	202 ± 12.96	189.86 ± 15.03	219.5 ± 9.75	193.75 ± 11.74**	146.2 ± 6.42***
	9w	387.56 ± 13.57	257.86 ± 17.28	301.11 ± 10.98	257.31 ±13.72***	223.47 ± 11.01***
BUN (mg/dL)	4w	15.58 ± 1.28	15.06 ± 2.46	17.79 ± 4.25	17.3 ± 2.68	15.3 ± 0.88
	6.5w	15.19 ± 1.58	15.73 ± 2.36	25.35 ± 2.58	22.38 ± 3.26*	14.4 ± 1.81***
	9w	17.37 ± 2.02	15.49 ± 1.43	40.58 ± 2.4	31.76 ± 2.39***	15.52 ± 1.8***
Serum creatinine (mg/dL)	4w	0.4 ± 0.04	0.39 ±0.01	0.39 ± 0.02	0.38 ± 0.01	0.38 ± 0.01
	6.5w	0.41 ± 0.02	0.4 ± 0.02	0.4 ± 0.02	0.41 ± 0.03	0.42 ± 0.06
	9w	0.44 ± 0.02	0.44 ± 0.02	0.59 ± 0.04	0.54 ± 0.04**	0.44 ± 0.03***
Urinary albumin/creatinine (mg/mmol)	9w	1.88 ± 0.83	2.49 ± 1.18	45.22 ± 20.83	14.06 ± 9.78***	2.06 ± 0.69***

2K/TBW% denotes two kidneys weight as a percent of the total body weight.

* P< 0.05, ** P<0.01, ***P<0.001 vs. Cy/+ Vehicle group

Table S2 NVP-BEZ235 effect on kidney morphology in male Han:SPRD rats

	+/+ Vehicle	*+/+ NVP-low*	*Cy/+ Vehicle*	*Cy/+ NVP-low*	*Cy/+ NVP-high*
Cystic index (%) (n)	3.93 ± 1.85 (5)	3.53 ± 1.52 (6)	32.86 ± 7.1 (7)	20.8 ± 4***(8)	13.18 ± 4.03***(5)
Ki-67 positive nuclear index (%) (n)	1.57 ± 0.57 (4)	1.15 ± 0.43 (5)	9.7 ± 4.36 (8)	4.41 ± 0.76**(6)	2.14 ± 0.46***(5)
Fibrosis index (%) (n)	8.71 ± 1.51 (3)	8.99 ± 6.29 (5)	24.79 ± 8.15 (8)	17.47 ± 4.95 (8)	12.27 ± 3.69*(5)

*P< 0.05, ** P<0.01, ***P<0.001 vs. Cy/+ Vehicle group

Table S3 NVP-BEZ235 and everolimus effect on polycystic kidney disease progression in male Han:SPRD rats

	Treatment time (weeks of age)	*2K/TBW*	*BUN (mg/dL)*	*Serum creatinine (mg/dL)*	*Cystic index (%)*
Cy/+ NVP-high (n=5)	4w ~ 9w	1.28 ± 0.26	15.52 ± 1.80**	0.44 ± 0.03*	13.18 ± 4.03*
Cy/+ everolimus (n=6)	4w ~ 9w	1.18 ± 0.09	25.30 ± 4.53	0.54 ± 0.08	22.33 ± 6.11

*P< 0.05, ** P<0.01 vs. Cy/+ everolimus group

Table S4 NVP-BEZ235 and sirolimus effect on the development of ADPKD in *Pkd1* conditional knockout mice.

	V- **n=8**	***N-*** **n=8**	***S-*** **n=5**	***V+*** **n=10**	***N+*** **n=7**	***LN+*** **n=7**	***S+*** **n=5**
Total body weight (g)	17.86 ± 1.51	16.55 ± 1.29	15.3 ± 2.6	10.17 ± 2.37	15.66 ± 1.33***	8.59 ± 1.96	9.56 ± 1.29
2K/TBW%	1.25 ± 0.1	1.17 ± 0.07	1.25 ± 0.1	8.06 ± 2.74	2.71 ± 0.67***	5.56 ± 1.05***	3.46 ± 1.41*
BUN (mg/dL)	21.5 ± 2.62	21.88 ± 3.91	20.8 ± 4.21	276.1 ± 180.97	63.57 ± 31.5***	225.4 ± 106.32	130 ± 124.29*
Serum Creatinine (mg/dL)	0.11 ± 0.01	0.13 ± 0.02	0.09 ± 0.01	0.61 ± 0.8	0.2 ± 0.05***	0.51 ± 0.23	0.28 ± 0.24*

2K /TBW% denotes two kidneys weight as a percent of the total body weight.

* P< 0.05, ** P<0.01, ***P<0.001 vs. V+ group

Table S5 NVP-BEZ235 and sirolimus effect on the development of ADPKD in *Pkd1* conditional knockout mice.

	V-	***N-***	***S-***	***V+***	***N+***	***LN+***	***S+***
Cystic Index (%) (n)	2.81 ± 0.77 (5)	2.66 ± 0.53 (5)	1.65 ± 0.42 (5)	51.31 ± 9.96 (8)	22.07 ± 8.87***(7)	50.39 ± 6.34 (6)	37.16 ± 5.57* (5)
Ki-67 positive nuclear index (%) (n)	1.46 ± 0.82 (5)	1.75 ± 1.01 (5)	3.32 ± 1.25 (5)	7.38 ± 2.94 (5)	2.79 ± 1.79*(7)	7.06 ± 3.54***(6)	5.15 ± 2.82*(5)
Fibrosis index (%) (n)	0.13 ± 0.06 (5)	0.41 ± 0.58 (5)	0.22 ± 0.04 (5)	11.11 ± 6.52 (5)	1.45 ± 0.83***(6)	4.69 ± 1.07**(6)	3.14 ± 0.87***(5)

* P< 0.05, ** P<0.01, ***P<0.001 vs. V+ group

Table S6 Plasma concentration of NVP-BEZ235 in male Han:SPRD rats

	Day 25 (µmol/l)*	*Day 28** (µmol/l)*
+/+ (n=3)	2.70 ± 1.48	3.17 ± 1.67
Cy/+ (n=3)	3.12 ± 0.13	2.76 ± 1.87

* Plasma obtained from NVP-BEZ235 naïve rats, aged 25 days, 1h after 50 mg/kg NVP-BEZ235 was applied by gavage.
** Plasma obtained from rats, aged 28 days, treated for 4 days with 50 mg/kg/day NVP-BEZ235 by gavage,1h after 50 mg/kg NVP-BEZ235 was applied by gavage.

Table S7 NVP-BEZ235 and sirolimus effect on autosomal recessive polycystic kidney disease progression in male PCK rats.

	V n=5	**N n=6**	**S n=5**
Total body weight (g)	182.8 ± 18.38	113.33 ± 7.158***	123.6 ± 12.34***
2K/TBW%	1.13 ± 0.03	1.13 ± 0.05	1.22 ± 0.6*
BUN (mg/dL)	9.8 ± 0.84	9.67 ± 2.88	11.6 ± 2.88
Serum Creatinine (mg/dL)	0.19 ± 0.01	0.19 ± 0.01	0.26 ± 0.03***
Cystic Index (%)	6.95 ± 2.41	1.72 ± 0.55***	2.74 ± 1.8**
Fibrosis index (%)	3.38 ± 0.95	4.12 ± 1.92	3.62 ± 2.07

* P< 0.05, ** P<0.01, ***P<0.001 vs. V group

11 Discussion and Conclusions

The insight of polycystic kidney disease molecular mechanisms promoting disease progression makes is it possible to elucidate therapeutic strategies for preclinical studies and clinical trials in PKD. Since PKD has been described as “neoplasm in disguise”, several anti-cell proliferation drugs have been tested in animal models and, some of them were shown to be effective and to be of potential value for the treatment of PKD. These include compounds inhibiting the following molecules: mTOR, CDK, MEK, EGF receptor and Src kinase [76-78 85 119, 120].

Targeting mTOR by rapamycin (sirolimus) and its analog everolimus for PKD treatment have been studies extensively in the recent years. mTOR inhibitor-based treatment decreased renal cyst size, reduced two kidney/body weight ratio and improved renal function in PKD animal models [76-78]. However, subsequent clinical trials published in the *New England Journal of Medicine* reported that mTOR inhibitors did not halt ADPKD progression of [5 6]. *Walz et al.* randomly assigned 433 patients with ADPKD and stages 1-3 chronic kidney disease to receive either everolimus (2.5 mg twice daily) or placebo for 2 years. Among patients receiving everolimus kidney and in cyst growth was lower in the first study year compared to placebo treated subjects, however the difference in cyst volume between the two groups was not statistically significant after 2 years treatment. The total kidney volume only had transient statistical significance after the first year and was no longer significant after the second year. The study by *Serra et al.* randomly assigned 100 patients with ADPKD and normal or mildly impaired renal function to sirolimus (2 mg

daily) or placebo for 18 months. At the end of the study period, total kidney volume was similar in the sirolimus group and the placebo group.

These disappointing results from these two clinical trials contrast with the positive findings from preclinical research. Some experts have argued that the time of treatment initiation was at late disease course (CDK 1-3 stages) and thus renal cyst formation and associated kidney damage was irreversible, particularly in the trial of Walz et al. The lack of a long-term beneficial treatment effect might be also be due to the high drop-out rate due to the drug-related adverse effects. The failure in the trial of *Serra et al.* might be due to the relatively low dose of sirolimus given to patients, which was could have been inadequate to achieve effective mTOR inhibition in renal cyst tissue.

In my PhD thesis, I explored alternative explanations for the failure of mTOR treatment in clinical trials: 1) The upstream regulator PLD/PA modulates the mTOR signaling pathway in ADPKD, which partly contribute to mTOR inhibitor-resistance in ADPKD. 2) Blocking mTOR could up-regulate the activation of the IRS1/PI3K-dependent pathway, which might counteract the drug effect in ADPKD.

11.1 PLD/PA and mTOR signaling pathways in PKD

In the first study, I investigated the PLD/PA signaling pathway in PKD. Although the mTOR signaling pathway plays a key role in PKD, little is known about the impact of PLD/PA signaling on PKD. Hyper-activation of PLD/PA signaling has been observed in human carcinomas, including ovary, breast, colon and kidney cancer, which all associate with high cell proliferation [9, 10]. I

found that the endogenous level of PLD activity was increased in all investigated animal and human PKD cells compared with control cells. A similar finding has been reported in studies with human carcinomas. Deregulated mTOR pathway activation might be mediated partly by increased PLD/PA signaling in PKD cells.

Targeting aberrant PLD/PA signaling by small molecule inhibitors reduced cell proliferation. It should be noticed that PLD inhibitors had an anti-proliferative effects on both PKD and normal renal tubular epithelial cells, and this might be due to cytotoxicity. Considering that disease development is similar to neoplastic disease, targeting cyst development through the PLD/PA pathway with PLD inhibitors needs further study in PKD animal models to evaluate the benefits and adverse effects. Interestingly, targeting PLD/PA signaling by PLD inhibitors induced autophagy. Autophagy is a catabolic process for the degradation of dysfunctional proteins through an autophagosomal-lysosomal pathway. Autophagy plays an important role in various aspects of cell physiology, especially cell survival during nutrient or energy limitation [121]. Autophagy can also trigger cell death and impair cellular functions in other contexts depending on the cellular environment, the nature and intensity of the stimulus, and the levels of autophagy [122]. However, our study was not designed to study whether autophagy induced by PLD inhibitors represents an autophagic process of cell death or whether it is a self-protective mechanism against cellular stress. Further studies are necessary to assess the role of autophagy in PKD, including methods to promote or inhibit autophagy via pharmacological and genetic manipulations.

The PLD/PA pathway plays a role as an upstream regulator of the mTOR signaling pathway. PA, as one of the products of PLD, is required for the stability of mTORC1 and mTORC2 and modulates the kinase activity of both complexes. PA binds with mTOR in a competitive manner with rapamycin [7 8]. We showed that blocking the PLD activity by PLD inhibitors and by the "alcohol trap" assay, which inhibited PLD activity by producing phosphatidyl alcohols instead of PA, decreased activation of the mTOR signaling pathway. However, exogenous PA could stimulate mTOR activity via increasing the activation of readouts of the mTOR pathway. These observations suggest that PLD/PA modulate the mTOR pathway in PKD cells.

Since PLD/PA interact with mTOR complexes by competing with the rapamycin binding at the level of the FKBP12/rapamycin domain [7 8], as a consequence, elevated PLD activity confers rapamycin resistance in PKD, which might partly explain the failure of clinical trials. In our study, combining mTOR and PLD inhibitors reduced the rapamycin resistance of PKD cells. Rapamycin-based therapeutics combined with targeting the PLD/PA pathway could be more effective for PKD treatment.

In summary, our data revealed an elevated PLD activity in PKD cells. The mTOR signaling pathway was modulated in a PLD/PA-dependent way. Targeting PLD blocked cell proliferation, decreased mTOR signaling and induced autophagy formation. Combination of mTOR and PLD inhibitors has a synergistic effect on retarding cell proliferation and blocking mTOR pathway. Targeting PLD may provide a new potential therapy for PKD.

11.2 Multi-target mTOR signaling pathway and its feedback loop in PKD

Targeting mTOR leads to a significant improvement of renal function and reduction in renal cyst volume in a variety of ADPKD animal models. However, two subsequent clinical trials failed to show significant clinical benefits both in early and advanced stages [5, 6]. This might be due to the fact that the mTOR signaling pathway is connected to a dual negative feedback loop, the PI3K-dependent pathway, which has been described in a lot of human cancers [11-13]. In our study, I showed that inhibition of the mTOR pathway by the mTOR inhibitors rapamycin or everolimus lead to compensatory upregulation of the PI3K-dependent pathway, diverting signals towards the Akt and ERK pathways in both human ADPKD patients and animal model in vivo and in vitro. This negative feedback loop might abolish the treatment effect of mTOR inhibitors in PKD. The resistance to mTOR inhibitor treatment may provide us a potential therapeutic strategy: multi-target treatment in PKD.

The activity of the mTOR inhibitor was monitored via the downstream effector S6K in human PBMCs and tumor tissue. It has been reported that there is a direct-link for S6K inhibition between in tumors and in PBMCs in a rat model [109]. In our research, we found decreased activation of S6, the readout of mTOR and S6K, meanwhile upregulated activation of ERK in PBMCs from patients of the Swiss ADPKD cohort who received either sirolimus or placebo for 6 months (Western blot analysis). Although the effect of sirolimus (2 mg/day) did not benefit the ADPKD patients, it affected the mTOR signaling and triggered the negative feedback loops (PI3K/Ras/ERK pathways) in

PBMCs in the treatment group. This suggested to us that the compensatory feedback loop stimulated by blockade of mTOR plays a role in mTOR-based treatment for ADPKD, although we observed one exceptional ADPKD patients who downregulated PI3K/Akt pathway in the sirolimus group. The activation of the dual feedback loop (PI3K/Akt and PI3K/Ras/ERK pathways) upon everolimus treatment was also found in Han:SPRD rats both *in vitro* and *in vivo*. These results hint to a complex molecular pathway network which could be related to the variable genetic background in ADPKD patients.

In my study, multi-target inhibition of mTOR and its feedback loop (PI3K-dependent pathways) either by a combination of mTOR, Akt and ERK inhibitors or by using the dual mTOR/PI3K inhibitor NVP-BEZ235 was more effective than mTOR inhibitors alone *in vitro* and *in vivo*. Although everolimus (3 mg/kg/day) significantly improved the renal function, the high dose of NVP-BEZ235 (50 mg/kg/day) dramatically preserved the renal function in Cy/+ group to a level which was similar to the wild-type group. Furthermore, NVP-BEZ235 showed an improved effect on cyst formation and improved renal function compared with sirolimus in *Pkd1* conditional knock out mice. Further studies suggested that mTOR and one of the dual feedback loop PI3K/Akt pathways are the primary targets responsible for the treatment effects, and that the PI3K/ERK pathway was indirectly affected by chronic treatment. To be noted, there is no effect on any cellular signaling pathway analyzed in wild-type treated kidneys in both mTOR inhibitor and NVP-BEZ235 group, suggesting the limitation of drug effect only to cystic kidneys.

Dose- and time-dependence of NVP-BEZ235 was demonstrated in Han:SPRD rats and *Pkd1* conditional knock out mice. NVP-BEZ235 treatment

reduced 2K/TBW ratios in the early disease stage from 4 to 9 weeks of age in Han;SPRD rats in a dose-dependent way. In the *Pkd1* conditional knock out mice, drug intervention timing just one day after tamoxifen induced-Pkd1 inactivation, retarded the cyst formation and preserved renal function. However, when the treatment was initiated at a more advanced stage of disease (after weaning), drug treatment lacked significant effects on cyst formation. Our data indicate that multi-target treatment may work effectively against cyst formation and disease progression when used at a suitable dosage and with adequate timing.

As we known, there is a wide range of adverse effect for mTOR inhibitor [123, 124]. Similar to mTOR inhibitors, adverse effects of NVP-BEZ235 are a concern for long-term treatment. Although we found there's a reduction in body weight in both low- and high-dose groups, NVP-BEZ235 was generally well tolerated in Han:SPRD rats. Recently, NVP-BEZ235 was shown a well tolerated in phase I/II clinical studies for patients with advanced solid malignancies (www.clinicaltrials.gov).

In summary, we observed that blockage of the mTOR pathway could lead to compensatory up-regulation of the PI3K-dependent pathway, either towards the PI3K/Akt or to the PI3K/ERK pathway, possibly depending on the genetic background in ADPKD. Whereas mTOR and dual mTOR/PI3K inhibitors both have efficacy on blocking ADPKD progression, the dual mTOR/PI3K inhibitor has greater effect in rat and mouse ADPKD. My studies suggest that multi-target mTOR and its dual feedback loop PI3K-dependent pathway by NVP-BEZ235 result in inhibition of mTOR and PI3K/Akt pathway, ultimately leading to decreased activity of the PI3K/ERK pathway. Taken together, these

findings demonstrate that multi-target therapy is an effective therapeutic strategy for ADPKD.

12 Outlook

Although the promising results in preclinical animal models, the results from clinical trials by using sirolimus or everolimus are disappointed [5 6]. This PhD thesis focused on elucidating mechanisms explaining these disappointing results from clinical trials, and setup new therapeutic approaches for ADPKD treatment.

For the first study, pharmacological and genetic approaches will be necessary to examine the role of isoforms PLD1 and PLD2 in ADPKD. Since PLD modulates mTOR activation, it is interesting to investigate the subcellular localization of PLD isoforms and mTOR in PKD cells. Autophagy is a catabolic process for the degradation of dysfunctional proteins through an autophagosomal-lysosomal pathway [121]. However, spare data are available on the impact of autophagy on PKD. Thus, it would be necessary to study the role of autophagy in PKD via a series of methods. Furthermore, we need to elucidate whether autophagy induced by PLD inhibitors represents an autophagic process of cell death or whether it is a self-protective mechanism against cellular stress. Targeting PLD could have an anti-proliferative effect in selective PKD cells, thus it is essential to examine the effect by using PLD inhibitors in PKD animal models in future studies.

For the second study, I suggest further studies to optimize the therapeutic strategy of multi-target treatment by using NVP-BEZ235 for ADPKD. We used two ADPKD animal models, a non-orthologous slowly progressive model

(Han:SPRD rats) and an orthologous rapidly progressive model (*Pkd1* conditional knock out mice using tamoxifen-induced *Pkd1* inactivation at P12), to investigate the effect of 5 weeks or 24 days treatment of NVP-BEZ235, respectively. It remains to be shown whether long term treatment with NVP-BEZ235 slows disease progression in Han:SPRD rats and *Pkd1* conditional knock out mice (i.e. tamoxifen-induced *Pkd1* inactivation after P13). Furthermore, a combined treatment with different compounds blocking simultaneously or sequentially alternative signaling pathways besides mTOR and its feedback loop may be of interest.

13 References

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14 Appendix

14.1 Co-Authorships

http://www.kidney-international.org basic research

Targeting of sodium–glucose cotransporters with phlorizin inhibits polycystic kidney disease progression in Han:SPRD rats

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Renal tubular epithelial cell proliferation and transepithelial cyst fluid secretion are key features in the progression of polycystic kidney disease (PKD). As the role of the apical renal sodium–glucose cotransporters in these processes is not known, we tested whether phlorizin inhibits cyst growth and delays renal disease progression in a rat model of PKD. Glycosuria was induced by subcutaneous injection of phlorizin in male heterozygous (Cy/+) and wild-type Han:SPRD rats. Phlorizin induced immediate and sustained glycosuria and osmotic diuresis in these rats. Cy/+ rats treated with phlorizin for 5 weeks showed a significant increase in creatinine clearance, a lower 2-kidneys/body weight ratio, a lower renal cyst index, and reduced urinary albumin excretion as compared with vehicle-treated Cy/+ rats. Measurement of Ki67 staining found significantly lower cell proliferation in dilated tubules and cysts of Cy/+ rats treated with phlorizin, as well as a marked inhibition of the activated MAP kinase pathway. In contrast, the mTOR pathway remained unaltered. Phlorizin dose dependently inhibited MAP kinase in cultured tubular epithelial cells from Cy/+ rats. Thus, long-term treatment with phlorizin significantly inhibits cystic disease progression in a rat model of PKD. Hence, induction of glycosuria and osmotic diuresis (glycuresis) by renal sodium-glucose cotransporters inhibition could have a therapeutic effect in polycystic kidney disease.

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the development of innumerable renal cysts which originate from the tubular epithelium of various nephron segments.[1] Cystogenesis in ADPKD reflects epithelial dedifferentiation, increased proliferation, and abnormal fluid secretion.[2] The compression of healthy adjacent parenchyma by the expanding cysts leads to progressive renal failure, with more than 50% of the patients reaching end-stage renal disease during their lifetime.[3] Insights into the pathophysiological processes that govern cyst development led to a growing number of drug candidates in ADPKD. In particular, several drugs targeting epithelial cell proliferation or the transport processes that contribute to intracystic fluid secretion have recently been proposed. These drugs include mammalian target of rapamycin (mTOR) inhibitors, somatostatin, and vasopressin type 2 receptor antagonists.[4,5]

The dihydrochalcone phlorizin is a natural product and dietary constituent which is found in a number of fruit trees.[6] For decades, it has been extensively used as a tool for physiological research. Phlorizin's principal pharmacological action is to produce renal glycosuria and—to a lesser degree—to block intestinal glucose absorption through inhibition of the Na^+–glucose cotransporters (SGLTs) located apically in the proximal tubules of the kidneys and the small intestinal mucosa. The administration of phlorizin to experimental animal models results in profound inhibition of the renal SGLTs in renal proximal tubules, resulting in marked glycosuria.[6]

To the best of our knowledge, the role of SGLTs in the pathogenesis of polycystic kidney disease (PKD) has never been examined. We hypothesized that induction of glycosuria and osmotic diuresis by inhibiting renal SGLTs could slow cystogenesis and renal disease progression. To test this

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Low-Dose Oral Sirolimus and the Risk of Menstrual-Cycle Disturbances and Ovarian Cysts: Analysis of the Randomized Controlled SUISSE ADPKD Trial

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Abstract

Sirolimus has been approved for clinical use in non proliferative and proliferative disorders. It inhibits the mammalian target of rapamycin (mTOR) signaling pathway which is also known to regulate ovarian morphology and function. Preliminary observational data suggest the potential for ovarian toxicity but this issue has not been studied in randomized controlled trials. We reviewed the self-reported occurrence of menstrual cycle disturbances and the appearance of ovarian cysts *post hoc* in an open label randomized controlled phase II trial conducted at the University Hospital Zürich between March 2006 and March 2010. Adult females with autosomal dominant polycystic kidney disease, an inherited kidney disease not known to affect ovarian morphology and function, were treated with 1.3 to 1.5 mg sirolimus per day for a median of 19 months (N = 21) or standard care (N = 18). Sirolimus increased the risk of both oligoamenorrhea (hazard ratio [HR] 4.3, 95% confidence interval [CI] 1.1 to 29) and ovarian cysts (HR 4.4, CI 1.1 to 26); one patient was cystectomized five months after starting treatment with sirolimus. We also studied mechanisms of sirolimus-associated ovarian toxicity in rats. Sirolimus amplified signaling in rat ovarian follicles through the pro-proliferative phosphatidylinositol 3-kinase pathway. Low dose oral sirolimus increases the risk of menstrual cycle disturbances and ovarian cysts and monitoring of sirolimus-associated ovarian toxicity is warranted and might guide clinical practice with mammalian target of rapamycin inhibitors.

Trial Registration: ClinicalTrials.gov NCT00346918

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Introduction

Sirolimus (Rapamune, Pfizer, New York, NY, USA) is a potent immunosuppressive and anti-proliferative drug which blocks the mammalian target of rapamycin (mTOR). MTOR is a key regulatory kinase which is also known to regulate ovarian function [1]. The drug has been approved by both the US Food and Drug Administration and the European Medicines Agency for the prevention of renal allograft rejection.

Most of our knowledge regarding sirolimus toxicity has been derived from kidney transplant efficacy trials. However there were no reports of menstrual cycle disturbances and ovarian cyst formation in three large clinical trials [2,3,4], although these adverse events were reported in three case series [5,6,7]. Ovarian dysfunction is difficult to recognize in clinical trials: symptoms are often non-specific and can be wrongly attributed to concomitant medication or comorbidities [8].

Aside from organ transplantation, sirolimus is being assessed for clinical effectiveness in several cancers and in other proliferative disorders, including autosomal dominant polycystic kidney disease (ADPKD). ADPKD is characterized by the growth of kidney cysts; the disease itself is not known to affect ovarian morphology and function [9,10,11]. Although sirolimus shows promise in rodent polycystic kidney disease models [12,13,14,15,16,17], 18 months treatment with sirolimus did not slow the growth of kidney cysts in adults with ADPKD [18].

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Anti-VEGF antibody treatment accelerates polycystic kidney disease

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Raina S, Honer M, Krämer SD, Liu Y, Wang X, Segerer S, Wüthrich RP, Serra AL. Anti-VEGF antibody treatment accelerates polycystic kidney disease. *Am J Physiol Renal Physiol* 301: F773–F783, 2011. First published June 15, 2011; doi:10.1152/ajprenal.00058.2011.— Polycystic kidney growth implies expansion of the vasculature, suggesting that vascular endothelial growth factor (VEGF)-dependent processes play a critical role and that VEGF is a putative therapeutic target. Whether an anti-VEGF antibody improves renal cystic disease has not been determined. We administrated 5 mg/kg B20.4.1, an anti-VEGF-A antibody, or vehicle intraperitoneally twice weekly to 4-wk-old male normal (+/+) and cystic (Cy/+) Han:SPRD rats for 6 wk. Renal function, urinary protein excretion, organ/body weight ratios, cyst volume, tubular epithelial cell (TEC) proliferation, renal VEGF, hypoxia-inducible factor (HIF)-1α and -2α expression, renal histology, and kidney hypoxia visualized by [^{18}F]fluoromisonidazole positron emission tomography were assessed. The treated compared with untreated +/+ rats had lower TEC proliferation rates, whereas Cy/+ rats receiving B20.4.1 displayed an increased proximal TEC proliferation rate, causing enhanced cyst and kidney growth. The +/+ and Cy/+ rats receiving B20.4.1 had severe renal failure and extensive glomerular damage. Proteinuria, which was highest in anti-VEGF-treated Cy/+ and lowest in untreated normal littermates, was positively correlated with renal HIF-1α and negatively correlated with VEGF expression. The untreated Cy/+ vs. +/+ rats had higher overall [^{18}F]fluoromisonidazole uptake. The +/+ rats receiving B20.4.1 vs. untreated had increased [^{18}F]fluoromisonidazole uptake, whereas the uptake was unchanged among treated vs. untreated Cy/+ animals. In conclusion, B20.4.1 caused an exaggerated cystic response of the proximal tubules in cystic rats and severe kidney injury that was associated with low renal VEGF and high HIF-1α levels. Anti-VEGF drug therapy may therefore not be a treatment option for polycystic kidney disease.

angiogenesis; Han:SPRD rat; cyst growth

AUTOSOMAL DOMINANT POLYCYSTIC kidney disease (ADPKD) is the most common hereditary kidney disease, resulting in progressive renal failure and end-stage renal disease in adulthood (37). Continuous cyst growth starts already in utero and is mediated by increased tubular epithelial cell proliferation. Kidney size increases from 150–200 cm^3 in adolescence to >1,000 cm^3/kidney in young adults (17, 31). Prominent cyst growth in ADPKD patients indicates fundamental defects in the regulation of proliferation, apoptosis, and neoangiogenesis (36).

Polycystic kidney growth implies expansion of the vasculature to supply the growing cysts with oxygen and nutrients (40). Further cyst expansion results in regional hypoxia and consecutive activation and upregulation of the hypoxia-inducible factor (HIF) pathway (4). HIFs are heterodimers composed of a constitutive β-subunit (HIF-β) and oxygen dependent α-subunits (HIF-1α and -2α) (4, 28). HIF-α is degraded in the presence of molecular oxygen, whereas during hypoxia HIF-α is stabilized and translocated to the nucleus, where it heterodimerizes with HIF-β, binds to the hypoxia-response element, and activates the transcription of several genes, including vascular endothelial growth factor (VEGF) (12).

VEGF or VEGF-A, an endothelial cell-specific growth factor, is a downstream effector of HIFs and plays a critical role in promoting angiogenesis (16, 19). VEGF exerts its biological effect through interaction with two receptor tyrosine kinases, VEGF receptor 1 (VEGFR1, also known as Flt-1) and VEGF receptor 2 (VEGFR2, also known as KDR/Flk-1). In the kidney, podocytes and tubular epithelial cells primarily express VEGF, whereas VEGF receptors are found in the mesangium, podocytes, and peritubular capillaries (4).

A previous study has shown that VEGF receptor blockade by ribozymes reduced cyst growth in the Han:SPRD rat, an animal model for polycystic kidney disease (33), indicating that VEGF-dependent processes play a critical role in cyst growth (1, 33) and that VEGF may be a putative therapeutic target. Whether an anti-VEGF antibody improves renal cystic disease has not been determined. Thus we administrated the anti-VEGF-A antibody B20.4.1 to Han:SPRD rats to test whether this treatment retards polycystic kidney disease progression. In addition, we visualized kidney hypoxia by using a functional imaging technique: [^{18}F]fluoromisonidazole positron emission tomography ([^{18}F]FMISO PET).

MATERIALS AND METHODS

Animals. The study was conducted in male heterozygous (Cy/+) Han:SPRD rats with cystic renal disease and in wild-type (+/+) Han:SPRD rats with normal kidneys. The Han:SPRD rat colony was established in our animal care facility from a litter which was initially obtained from the University of Missouri Rat Resource and Research Center (Columbia, MO). All experiments were performed according to protocols approved by the regulatory commission for animal studies, a local government agency. Rats had free access to tap water and standard rat chow.

Study drug. B20.4.1 is an anti-VEGF antibody which has been derived from synthetic antibody phage libraries. It selectively neutralizes all isoforms of VEGF-A, with no detectable activity against the related VEGF family ligands (20). Liang and colleagues (20) have shown that B20.4.1 and bevacizumab (rhuMAb-VEGF, Avastin) have similar binding affinities, specificities, and VEGF-A-blocking activities in vitro and in vivo (20). B20.4.1 twice weekly at an intraperitoneal (ip) dose of 5 mg/kg inhibited HM-7, A673, and HPAC tumor cell growth in Beige Nude XID mice, whereas lower doses (0.1 and 1 mg/kg) had no antitumor activity (20). Anti-VEGF antibody B20.4.1 and IgG vehicle were kindly provided by Genentech (South San Francisco, CA) and were stored at 4°C.

Experimental protocol. At *week 4*, rats were weaned and 5 mg/kg B20.4.1 (Cy/+, $n = 8$; +/+, $n = 6$) or vehicle (Cy/+, $n = 5$; +/+, $n = 6$) was administrated twice weekly ip until rats were eutha-

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ORIGINAL ARTICLE

The BH3-mimetic ABT-737 inhibits allogeneic immune responses

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ABT-737, allograft, apoptosis, Bcl-2, immunosuppression, transplantation.

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Conflicts of Interest
P.D.B. is an employee of Abbott, which developed and provided ABT-737. However, no financial sponsoring was received for this study and no conflict of interest exists for the other authors.

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Summary

Apoptosis controls the adaptive immune system through regulation of central and peripheral lymphocyte deletion. Therefore, substances that selectively interact with the intrinsic apoptosis pathway in lymphocytes offer unexplored opportunities to pharmacologically modulate the immune response. Here, we present evidence that the BH3-mimetic ABT-737 suppresses allogeneic immune responses. *In vitro*, ABT-737 prevented allogeneic T-cell activation, proliferation, and cytotoxicity by apoptosis induction, but without impairing the physiological functions of remaining viable T cells. *In vivo*, ABT-737 was highly selective for lymphoid cells and inhibited allogeneic T- and B-cell responses after skin transplantation. The immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose cyclosporine A, as shown by the induction of long-term skin graft survival without significant inflammatory infiltrates in 50% of the recipients in an MHC class I single antigen mismatched model. Thus, pharmacological targeting of Bcl-2 proteins represents a novel immunosuppressive approach to prevent rejection of solid organ allografts.

Introduction

The identification of alternative pharmacological targets to suppress allo-specific immune responses is a fundamental step in the development of new drugs aiming at the optimization of long-term outcome after solid organ transplantation [1]. It has been shown that the modulation of apoptosis in lymphocytes is responsible for central and peripheral repertoire selection [2,3] and controls the deletion of alloreactive lymphocytes in the induction of peripheral transplantation tolerance [4]. Therefore, targeting apoptosis pathways in lymphocytes represents a potential novel strategy for immunosuppression.

Two distinct but interconnected pathways regulate apoptosis in mammalian cells: the extrinsic pathway is mediated by death receptors at the cell surface (e.g. by Fas- or TNF-receptor), whereas the intrinsic (or mitochondrial) pathway is under the control of pro- and anti-apoptotic members of the Bcl-2 family [5]. Selective small-molecule Bcl-2 antagonists have rationally been developed for the treatment of tumors [6]. The Bcl-2 inhibitor ABT-737 and its orally bioavailable counterpart

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